Heterologous gene expression in *Saccharomyces cerevisiae:* Analysis of *Bacillus subtilis* β-glucanase and *Escherichia coli* β-glucuronidase.

Thesis
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I hereby declare that the research described within this thesis is based entirely upon my own work.

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ABSTRACT

The production of the *Bacillus subtilis* β-glucanase enzyme was examined in the yeast *Saccharomyces cerevisiae*. The ability of the bacterial signal peptide to translocate and secrete the enzyme was analysed and compared to the yeast α-factor signal peptide. A truncated version of the signal was also examined as was the effect of complete removal of the signal sequence. Glycosylation of the β-glucanase enzyme in the yeast secretory pathway was examined using both Western blots and activity gels and the effects of glycosylation on β-glucanase production was assessed. A second bacterial enzyme, β-glucuronidase, was also examined for its potential use as a reporter enzyme in yeast expression and secretion systems. This work included attempts to secrete the enzyme, the definition of its active site, investigation of its potential for use in translational fusion experiments and the generation of a polyclonal antibody to the *E.coli* β-glucuronidase enzyme.

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Abbreviations used in diagrams:

B : *Bam*H1
P : *Pst*1
H : *Hind*III
S : *Sal*1
C : *Cla*1
E : *Eco*R1
Sm: *Sma*1
D : *Dde*1
X : *Xba*1

'P' : promoter.
'T' : terminator.
Sp : signal peptide.

Other abbreviations

S.cerevisiae : *Saccharomyces cerevisiae*.
E.coli : *Escherichia coli*.
B.subtilis : *Bacillus subtilis*.
β-GUS : β-glucuronidase gene.

Amino acid codes:

A = Alanine.
D = Aspartic acid.
E = Glutamic acid.
L = Leucine.
K = Lysine.
H = Histidine.

CHAPTER 1
INTRODUCTION

(1.1) Heterologous protein production in yeast: an overview.

S.cerevisiae has many advantages as a host for the production of proteins of commercial value. The high cell densities reached in fermentations, using simple media, has made it an ideal organism for applications at an industrial level. In addition, the rapid progress being made in characterizing *S.cerevisiae* at biochemical and molecular levels and the ease with which the organism can be manipulated genetically, has greatly enhanced its potential in this respect.

There are a wide range of systems available for introducing, expressing and maintaining foreign genes in yeast, some of which are outlined below. The production and isolation of a particular protein however, goes beyond the successful maintenance and expression of the gene. A major problem encountered is insolubility of the gene product in the yeast cytoplasm. This problem is also associated with intracellular production in the bacterial host, *E.coli* and can result in the recovery of malfolded, biologically inactive material. Other factors such as protein instability and degradation can also affect recovery levels. Some of these problems and possible solutions are discussed in the remainder of this initial section. The potential of improving both recovery levels and product quality, by directing heterologous proteins through the secretory pathway is also examined.

(1.1.1) Yeast expression systems.

A number of vector systems are available for the introduction and maintenance of exogenous genes in *S.cerevisiae*. These include autonomously replicating plasmids which are maintained at high, medium or low copy numbers and integrating vectors whose copy number can also be controlled to a certain extent.

The most widely used autonomously replicating vectors are based on the native 2μm circle plasmid (Hartley and Donelson, 1980). These vectors include the 2μ origin and REP3 regions required for maintaining copy number and partitioning fidelity (Jayaram *et al* 1985). Copy number is controlled by the REP1, REP2 and FLP functions which are provided by endogenous 2μ circles in

cir^+ hosts (Wu *et al* 1987). 2μ based vectors are maintained at relatively high copy numbers (20-40 copies/cell) and stability is high under selective growth conditions. Autonomously replicating sequences (ARS), derived from chromosomal elements have also been included into vectors and although higher copy numbers are achieved, this is at the expense of stability and partitioning fidelity, even with selective growth conditions (Murray and Szostak, 1983).

Methods of increasing copy number and maintaining stability of 2μ based vectors have been developed. The introduction of the leucine defective (leu-d) gene as a selectable plasmid marker maintains a higher than average number of plasmid copies per cell. This is due to the inefficiency of the leu2 defective promoter which reduces transcription levels of the gene. High copy number is required to allow cell growth in leu2 auxotrophic strains (Erhart and Hollenberg, 1983). Along a similar line, the TPI1 (triose phosphate isomerase) gene from *Sz.pombe*, which is not expressed well in *S.cerevisiae*, has been introduced into vectors for propagation in *tpi*1 mutants. High copy number is maintained to ensure that adequate levels of TPI are produced to allow cell growth (Thim *et al*, 1986)

One copy of a plasmid can be stably maintained by the introduction of a yeast centromeric sequence (CEN) into an ARS or 2μ based plasmid. CEN plasmids are maintained at 1-2 copies per haploid genome and confer stability onto unstable vector systems such as ARS based plasmids (Tschumper and Carton, 1983). The advantages of maintaining low copy numbers come into play when for example, the protein of interest is toxic to the host.

More rigid control of copy number can be achieved by directing an expression cassette (ie. promoter and heterologous gene) into the yeast genome using integrating vectors (Smith *et al*, 1985). Integrating vectors lack replication origins and can be directed to any part of the genome by carrying at least one portion of a yeast gene to provide homology. In this way the heterologous sequences are maintained in the genome without the need for selective growth conditions. Multiple integration

events can be achieved by directing the vector to Ty elements, δ sequences or rDNA genes which are present in the genome at > 1 copy (Melnick *et al*, 1990; Fujii *et al*, 1990). It has been shown that some secreted proteins can be produced at higher levels and secreted with greater efficiencies when the expression/secretion cassette is integrated into the genome. Prochymosin and urinary plasminogen activator are secreted almost 4 times as efficiently under these conditions compared to levels secreted when expressed on autonomously replicating multicopy vectors (Smith *et al*, 1985; Melnick *et al*, 1990). This suggests that very high expression levels are not necessarily ideal for heterologous protein secretion.

The expression level of a gene can also be controlled by the choice of promoter. Constitutive promoters such as ADH1 (Ammerer, 1983), PGK1 (Hitzeman *et al* 1982) and GAPDH1 (Bitter and Egan, 1984) have been cloned, characterised and used for the expression of heterologous genes. These promoters transcribe their natural products at levels of >1% of total cell mRNA and protein. However, transcription of heterologous DNA in autonomously replicating vectors is not as efficient using these promoters as transcription of the natural gene. PGK1 for example is highly expressed in yeast, accounting for up to 5% of total cell protein and mRNA. Proteins produced from fusions to the cloned promoter sequence however result in much lower yields (Mellor *et al*, 1985). Even PGK produced in this manner is transcribed at lower than normal levels.

Inducible promoters are particularly useful in yeast expression systems in that high cell densities can be reached before the expression of the product is switched on. Many inducible promoters depend on the carbon source. The GAL1 and GAL10 promoters are tightly repressed in glucose medium but rapidly induced when galactose is introduced as a carbon source (Johnson and Davis, 1984). Changing the growth medium however, is not necessarily ideal in many industrial situations and the ADH2 promoter is more useful in this respect (Yu *et al*, 1989). ADH2 is turned off in the presence of glucose. The gradual depletion of glucose from the medium during cell growth can be

exploited to allow expression from this promoter to occur naturally, late in the growth cycle.

Transcriptional initiation from yeast promoter sequences usually occurs between 11 and 160 nucleotides from the translational initiation codon, (AUG). The average length of the 5' untranslated region is 52 nucleotides (Cigan and Donahue, 1987). Fusions to foreign genes however, usually results in lengthening the 5' non-coding region with the introduction of foreign sequences, often derived from restriction sites used in manipulating the various components of the expression cassette. Within the above range, the length of the leader region does not appear to affect translation efficiency of heterologous transcripts in yeast. However, the sequence content of this non-coding region can be of major importance.

Examination of leader sequences of highly expressed genes shows them to be largely void of secondary structure (Cigan and Donahue, 1987). The introduction of regions of dyad symmetry into the 5' region of HIS4 was shown to cause a reduction in translation to <5% of wild type levels (Cigan *et al*, 1988). Yeast appears to be sensitive even to single G and C nucleotides in this region and in the absence of secondary structure, the presence of Gs and Cs can have a detrimental effect on translation. This is thought to be due to intra and intermolecular interactions in this region of the transcript. The marked absence of Gs and abundance of As in the 5' untranslated leaders of yeast genes is a reflection of this effect (Cigan and Donahue, 1988).

Another feature affecting translation efficiency in yeast is the importance of the first AUG initiation codon. Studies examining preferred leader regions have shown that translation is initiated preferentially at the first AUG encountered by the ribosome. The introduction of out of frame AUG codons 5' of the initiator in HIS4 was found to reduce translation to less than 20% of normal levels (Donahue and Cigan, 1987). With the definition of ideal sequences arising out of the extensive analyses of Cigan and Donahue outlined briefly above, there is

definite potential for optimizing leader regions of foreign genes in order to maximize translation efficiency. Increased production levels of various foreign proteins in yeast has been observed when the 5' non-coding sequences are replaced either with yeast sequences or optimized leaders (Kniskern *et al*, 1986; Bitter and Egan, 1988).

(1.1.2) **Heterologous protein stability.**

A protein that can be produced in yeast cytoplasm in a soluble active form, can be recovered in quantities exceeding the levels produced in many secretion systems (Sabin *et al*, 1989). However, there are additional problems associated with intracellular expression and production of proteins apart from those already described. A major problem is instability of the foreign gene product in the yeast cytoplasm and this can depend on the nature of the N-terminus of the protein. The N-terminal formylated methionine residue of cytoplasmic proteins in *E.coli* is always removed by the action of MAP (methionine aminopeptidase) (Ben-Bassat *et al*. 1987). In eukaryotes, including *S.cerevisiae*, this methionine is not always removed and when it is retained, it may or may not be modified by acylation. Removal of the initiator methionine is dependant on the identity of the adjacent amino acid residue. Once exposed, this amino acid in turn may be acylated (Bradshaw, 1989). These modifications are often essential for biological activity of proteins. For proteins that are normally cytoplasmic residents, intracellular production in yeast will usually result in processing according to the general rules. However, secretory proteins rarely have N-terminal methionine residues in their mature sequences and may be processed incorrectly when produced in the cytoplasm.

For the production of proteins for pharmaceutic and therapeutic use, an authentic gene product identical to the naturally occurring counterpart is usually required. Removal of an unwanted initiator methionine can occur naturally *in vivo*. This can also be achieved by chemical treatment with cyanogen bromide or enzymatically using the cloned *E.coli* MAP protein.

In yeast, the identity of the penultimate residue then exposed can be vital in determining the stability of proteins in the cytoplasm and can be responsible for targeting a protein for degradation by the ubiquitin degradation pathway.

Ubiquitin is a 76 amino acid protein involved in the recognition system of protein degradation pathways in eukaryotic cells (Ozykaynak *et al.* 1987). Although the exact mechanism of its action is poorly understood, ubiquitin is known to form conjugates with a variety of intracellular proteins and targets abnormal or damaged proteins for proteolysis. Ubiquitin is processed by a specific protease that recognizes the carboxyl terminal Arg-Gly-Gly sequence. The specific processing of ubiquitin has been exploited to examine the effects of various N-terminal amino acids on protein stability in the yeast cytoplasm.

Ubiquitin was fused to the N-terminal of β-galactosidase with the Arg-Gly-Gly cleavage site separating the two proteins (Bachmair *et al*, 1986). Fusions were arranged so that different residues were present at the N-terminus of the β-galactosidase protein after processing by ubiquitinase. These fusions were efficiently processed by this enzyme and the processed β-galactosidase examined for stability. Amino acids Met, Ser, Gly, Val, Pro, Cys, Ala, Thr were identified as stabilizing residues, giving β-galactosidase a half-life of up to 30 hours. Amino acids Glu, Gln, Asp, Asn, Ile, Leu, Phe, Trp, Tyr, His, Lys, Arg were shown to be destabilizing when present at the N-terminal of β-galactosidase, exhibiting half-lives of 30 minutes or less.

The definition of this N-end rule has allowed the choice of appropriate fusions to maximize stability of heterologous proteins in yeast. The ubiquitin fusion system can be used to produce active stable proteins with authentic N-termini. Human γIFN (interferon) and α_1PI (proteinase inhibitor) have been produced in yeast using this system (Sabin *et al*, 1989). In some cases, increased levels of a heterologous protein have been reported when produced as a ubiquitin fusion (Ecker *et al*, 1989).

An additional problem associated with the recovery of heterologous proteins from the yeast cytoplasm, is degradation by the action of cellular proteases. This can present a problem, not only for cytoplasmically produced proteins, but also in assaying the intracellular pool of proteins directed to the secretory pathway. The complement of vacuolar proteases, particularly proteases A and B (PrA and PrB) and carboxypeptidases Y and S (CpY and CpS) are the major source of the proteolysis problem in yeast. These proteases complex with cytosolic inhibitors when released from the vacuole during cell lysis procedures. In many cases however, the nature of the lysis solutions and assaying procedures promote release of the proteases from their inhibitors, rendering them fully active (Jones, 1984). The presence of SDS, increased temperatures and pH can have this effect (Ulane and Cabib, 1976; Zubenko *et al*, 1979). Certain precautions, such as the use of protease inhibitors like PMSF and EDTA can alleviate the problem to some extent. However, a genetic approach to the protease problem has proved more successful.

PEP4 is the structural gene encoding the PrA precursor. This protein activates the other vacuolar proteases PrB and CpY on transfer into the vacuole (Ammerer *et al*, 1986). Disruption of PEP4 by the introduction of nonsense codon (*pep*4-3 mutants) has a pleiotropic effect on vacuolar hydrolases. PrA production is eliminated and the levels of PrB and CpY are significantly reduced. However, PrB can still cause problems even in a *pep*4 mutant. The *prb*1 mutation eliminating the PrB protein altogether, has also been isolated and the use of a double mutant (*pep*4-3 *prb*1) is recommended where proteolysis is a major concern.

(1.1.3) **Improving production by secretion.**

Many of the problems associated with intracellular production of heterologous proteins in yeast can be avoided by directing the protein through the secretory pathway. Producing proteins in the yeast culture medium, relatively free of contaminating host proteins, greatly eases purification procedures and is a

major advantage over the popular bacterial host, *E.coli*. In the reducing environment of the ER, disulphide bond formation occurs more readily than in the cytoplasm and this is of major importance for maintaining solubility and in some cases, biological activity. Protein glycosylation in the secretory pathway can contribute to product solubility and biological activity of mammalian proteins in particular. In addition, by generating the correct fusions, secretion can allow the generation of authentic N-termini and the secretory pathway can afford foreign proteins protection from the action of cellular proteases.

Two proteins, prochymosin and human serum albumin (HSA), are examples of success (Smith *et al*, 1985; Etcheverry *et al*, 1986). In both cases, attempts to produce the protein intracellularly in yeast resulted in low yields of largely insoluble and inactive material. Secretion however allowed soluble, correctly folded, fully active protein to be recovered. Directing the human growth hormone (hGH) through the secretory pathway has also led to increased recovery levels by up to 300 fold (Brake *et al*, 1984).

Although the secretion of foreign proteins in yeast is not without its own particular problems, many of which will become apparent, detailed characterization of the yeast secretory process has gone a long way in helping to overcome many of these. A better understanding of secretion has also been invaluable in working towards optimizing heterologous protein production in yeast.

Protein translocation across membrane structures is a process common to prokaryotic and eukaryotic cells. All cells produce proteins that must be transported from their site of synthesis to various subcellular organelles and compartments, or out into the cell medium, in order to perform their specific functions. Uncovering the molecular mechanisms involved in selective protein transport and sorting has been the subject of intensive research over the last 20 to 25 years. The rest of this review will attempt to cover the substantial progress that has been made in the area of protein transport through eukaryotic

secretory systems. Much of the information that has allowed characterization of the yeast secretory process has come from analysis of secretion in bacterial and mammalian cells. Emphasis will be placed on the developments made in eukaryotes and relevant aspects of the bacterial secretory process will also be discussed. Finally, a short summary of the initial successes in the area of heterologous protein secretion in *S.cerevisiae* is also included.

(1.2.0) **Introduction to the secretory process.**

Although bacterial cells do not have distinct organelles they are compartmentalised to a certain extent. In the gram negative bacterium *E.coli*, for example, the cytoplasm is surrounded by an inner and an outer membrane, separated by the periplasmic space. Proteins are transported from the cytoplasm to both membranes and to the periplasm by what is believed to be a common export pathway (Ito *et al.* 1981). Eukaryotic cells on the other hand, are compartmentalised to a much higher degree. To understand the complexities of the mechanisms that control eukaryotic protein transport, one must consider that apart from directing soluble proteins through the secretory pathway, the secretory process is also involved in cell surface assembly and in the assembly of subcellular organelles (Erikson and Blobel, 1985; Rome *et al.* 1979; Shore *et al.* 1979). In addition, proteins produced in the cytoplasm must be transported to the nucleus, the mitochondrion and chloroplasts, while others are inserted into cellular and subcellular membranes. The molecular mechanisms by which cells sort and traffic proteins to their various destinations are being gradually uncovered. The process however is far from fully understood.

Most of the initial information about protein translocation into and transport across membranes came from genetic evidence in prokaryotes (Benson *et al.* 1975) and from biochemical studies in eukaryotic cell free systems (Walter *et al.* 1984). Studies on protein localisation in these systems has allowed the identification of a large number of components involved in

the secretory process. A notable outcome of this work was the realisation that the mechanisms involved have been conserved to some extent between these groups. Bacteria have been shown to export eukaryotic secretory proteins (Talmadge *et al.* 1980) and in turn, eukaryotic cells can secrete certain bacterial proteins (Weidmann *et al.* 1984).

With eukaryotic cell free translation and translocation systems, extrapolation to *in vivo* situations is difficult. The lower eukaryote yeast, has a secretory system very similar to that of higher eukaryotic cells (Schekman *et al.* 1982). In contrast to mammalian cells it is easily manipulated genetically and therefore has the additional advantage of allowing *in vitro* assays to be backed up at a molecular level by the creation of mutants. For these reasons the potential of yeast as an organism to examine and identify components and steps involved in eukaryotic protein secretion has been realised. Several yeast genes have been cloned covering various steps and components involved in the secretory process and yeast has become a useful test system for translocation and transport analysis.

(1.2.1) **The secretory pathway of eukaryotic cells.**

The eukaryotic secretory pathway was defined by following the intracellular route taken through cells by pulse labeled proteins using electron microscope autoradiography and cell fractionation techniques (Jamieson and Palade, 1967a; 1967b; 1968). This pioneering work was carried out in pancreatic exocrine cells which are specialised for secretion. Secretory proteins were shown to be first sequestered into the lumen of the endoplasmic reticulum (ER). From there they enter the *cis* face of the Golgi apparatus *via* ER-membrane derived vesicles. The proteins are concentrated in secretory vesicles which are released from the *trans* side of the Golgi. These vesicles fuse with appropriate membranes, releasing the proteins into their correct subcellular location or out into the cell medium (Palade, 1975).

In yeast the secretory pathway was defined by the isolation of

a series of secretion defective mutants (Novick and Schekman, 1979). Yeast, unlike pancreatic cells is not specialised for secretion. Precursors are present in relatively low concentrations and the secretory organelles are fewer in number compared to secretory cells of higher eukaryotes. These features however, make yeast a sensitive system for examining protein secretion as the consequences of disruption of the secretory process are pronounced.

Cytological analyses have shown that secretion is limited to the growing bud surface of the yeast cell wall and is an essential function involved in cell growth (Linnemans *et al*, 1977). The first secretion defective (*sec*) mutants were isolated as conditional lethal growth mutants of *S.cerevisiae* (Novick and Schekman, 1979). These temperature sensitive mutants were screened for defects in protein secretion by assaying for a block in secretion of the invertase enzyme (Novick *et al* 1980). At the non-permissive temperature (37^0C) the block in secretion of invertase, an enzyme which is normally secreted to the periplasmic space, is accompanied by an intracellular accumulation of active enzyme. Another feature of these *sec* mutants is that they accumulate exaggerated secretory organelles at the stage in the pathway where the block occurs. This can be observed in electron micrograph thin sections of cells. Also, in most cases, the defect in secretion is reversible and normal growth and secretion is restored on returning the cells to the permissive temperature (25^0C). Initially, 192 of these mutants (classed as *sec*A) were isolated and were classified into 23 complementation groups.

Another set of mutants (*sec*B) were also isolated in these studies. These *sec*B mutants are temperature sensitive for growth but do not accumulate active secretory proteins at the non permissive temperature. *Sec*B mutants define four complementation groups and two in particular, *sec*53 and *sec*59, have been characterised in detail (Ferro-Novick *et al*. 1984). *Sec*53 and *sec*59 accumulate inactive forms of invertase and acid phosphatase (another yeast secretory protein) and the defect has been shown to affect the glycosylation process.

The generation of double secretory mutant strains and their characterization with regard to invertase accumulation and organelle morphology, allowed the order of events in the yeast secretory pathway to be defined (Novick *et al.* 1981). In theory, a double mutant will accumulate the intermediate prior to the first block encountered in the pathway. Using this approach, the yeast secretory pathway was shown to closely resemble that of higher eukaryotes. ER accumulating mutants are epistatic to Golgi and secretory vesicle mutants and in turn, Golgi mutants are epistatic to secretory vesicle mutants. Many of the mutants isolated in these studies and other isolated since, have been analysed in detail. In some cases the genes have been cloned, the protein products identified and functions assigned. Studies of the *sec* mutants and double mutants,plus work on the glycosylation of yeast secretory proteins and vacuolar mis-sorting mutants (Esmon *et al.* 1981; Stevens *et al.* 1982) can be combined to give a diagrammatic representation of the route taken by secretory proteins in yeast (Fig.1.1).

Figure (1.1) The secretory pathway of yeast as defined by *sec* mutant analysis (Schekman, 1982).

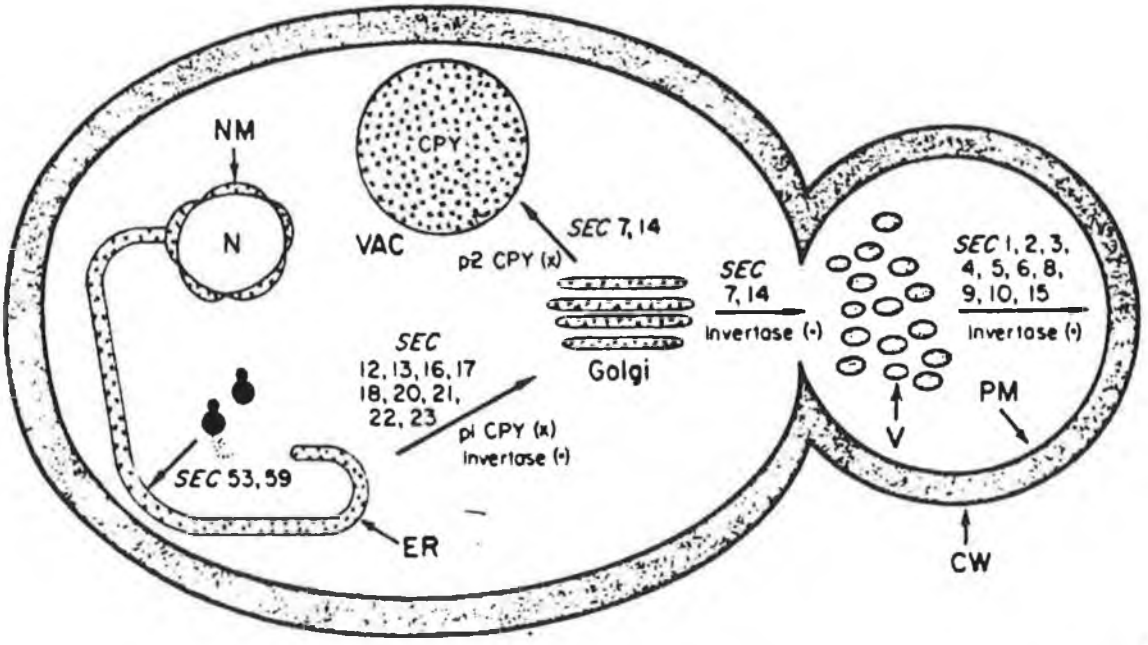


Fig. 1. Secretory and vacuolar protein transport pathways in yeast. N: nucleus; NM: nuclear membrane; ER: endoplasmic reticulum; SEC: wild-type gene; VAC: vacuole; V: vesicle; PM: plasma membrane; CW: cell wall; CPY: 61 Kd mature carboxypeptidase Y; pl CPY: a 67 Kd pro-enzyme form of CPY; p2 CPY: a 69 Kd pro-enzyme form of CPY.

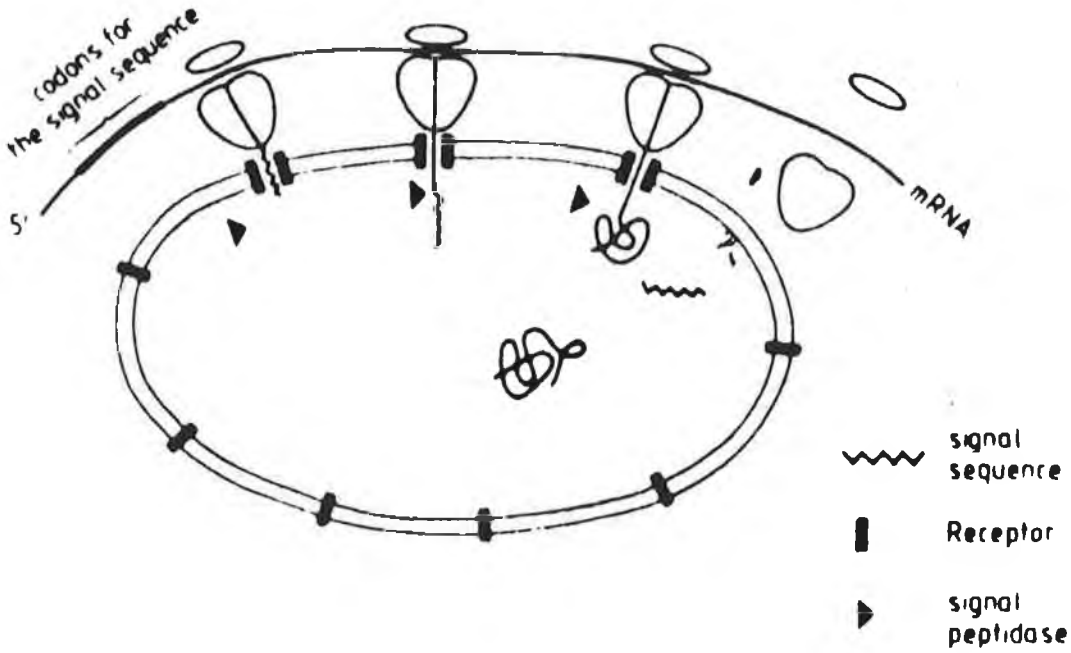
(1.2.2) **The signal hypothesis.**

The first suggestion that secretory proteins themselves contain information for selectivity in the export process came with the development of cell free translation systems (Milstein *et al.*1972; Schecter *et al.* 1975). Immunoglobulin light chains, when translated *in vitro*, were shown to be produced in a larger form than was finally secreted. These precursors had short amino terminal extensions and it was suggested that this series of amino acids might contain information that could direct the protein to the ER membrane.

This was later confirmed with the development of an assay that could directly measure protein transport across and interaction with membrane structures (Blobel and Dobberstein, 1975a; 1975b). In this assay, canine pancreatic microsomes were added to cell free translation systems and the translation of immunoglobulin light chains was examined. The microsomes, if present during protein synthesis, were shown to sequester newly made precursors of immunoglobulin light chains and process them to their correct size. These amino terminal extensions were termed signal peptides (also referred to as signal sequences or leader peptides).

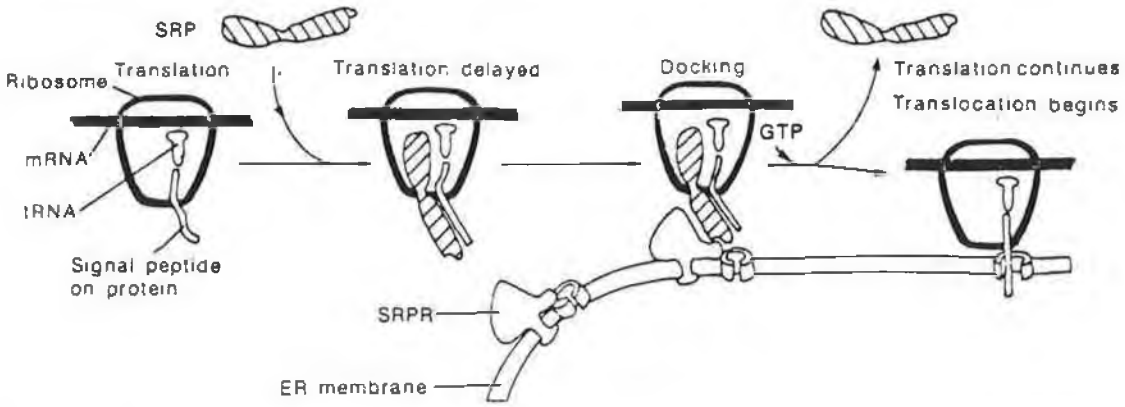
In 1975, Blobel and Dobberstein proposed the signal hypothesis. This is a model proposing the a series of molecular events accounting for the co-translational transfer of secretory proteins across the ER membrane. According to the early model, the signal peptide is the basis for selectivity of proteins for export through the secretory pathway. As the signal emerges from the ribosome, it is recognised and bound by specific receptors in the ER membrane, directing the nascent protein across the lipid bilayer *via* a putative pore or tunnel. The signal peptide is removed during or after transfer yielding the mature protein in the lumen of the ER (Fig.1.2).

Figure (1.2) The early signal hypothesis



The cell free transport assays developed by Blobel and Dobberstein allowed the isolation of some of the essential components of the transport machinery proposed in the early signal hypothesis. Using biochemical purification and fractionation techniques, two components were isolated, the signal recognition protein (srp) and srp receptor or docking protein (Walter and Blobel, 1980, 1981; Meyer and Dobberstein, 1980). Srp was shown to bind the signal peptide as it emerges from the ribosome putting a halt on further translation. It was proposed that the srp-polypeptide-ribosome complex then interacts with the membrane bound srp receptor, translation resumes, the signal peptide is cleaved and the protein is translocated (Fig.1.3). The srp and docking protein are released leaving the processed form of the secretory protein residing in the lumen of the ER. Models describing translocation into the ER are being continuously updated as more information about the components involved is uncovered. Recent developments are discussed in section 1.4.

Figure (1.3) The signal hypothesis.



Targeting of a growing protein chain to the endoplasmic reticulum (ER) membrane, mediated by signal recognition particle (SRP) and its receptor (SRPR).

(1.3) Characterizing eukaryotic and prokaryotic signal peptides.

After the isolation of the first eukaryotic signal peptide, it soon emerged that prokaryotic secretory proteins are also produced in precursor forms with signal sequences similar to eukaryotes (Inouye and Beckwith, 1977). The similarities between the two systems, despite the differences in complexities, soon became apparent. Eukaryotic and prokaryotic signals are functionally interchangeable. The *E.coli* secretory protein, β-lactamase, can be synthesised in a eukaryotic cell free translation system and is efficiently translocated into dog pancreatic microsomes (Muller *et al.* 1982). Srp has been shown to interact directly with this bacterial signal peptide effecting its translocation and when microinjected into *Xenopus* oocytes, it is efficiently processed and secreted into the cell medium (Weidmann *et al.* 1984).

This functional interchangeability is successful *in vivo* also. The *Bacillus* α-amylase enzyme, for example, is secreted in yeast using its own signal peptide (Ruohonen *et al.* 1987). Similarly in bacterial cells, some eukaryotic proteins can be secreted (Talmadge *et al.* 1980). Also, signal peptides can direct cytosolic proteins to the secretory pathway. The eukaryotic globin protein for example, when fused to the β-lactamase signal peptide is translocated into microsomal vesicles (Lingappa *et al.* 1984) and in *E.coli*, the LamB (receptor for phage lambda) signal peptide targets β-galactosidase to the cell membrane (Emr *et al.* 1980; Emr and Silhavy, 1980). Signal sequences therefore play an central role in protein export. They are necessary for directing proteins to the bacterial or ER membrane, a role that appears to be conserved to some extent between two evolutionarily diverse groups.

(1.3.1) Physical disection of signal peptides.

Although signal peptides perform conserved functions, systematic comparisons of amino acid sequences from prokaryotes and eukaryotes reveal no overall consensus sequences or

absolute amino acid requirements (von Heijne 1984a, 1984b; Perlman and Halvorsen, 1983). Furthermore, signal peptides differ in length as well as in amino acid composition. These comparisons however, reveal that all signal peptides conform to an overall tripartite structure of three structurally distinct domains : a positively charged amino terminal domain (N-domain), a central hydrophobic core (H-domain) and a polar carboxyl terminus (C-domain) which contains the cleavage site.
In a study comparing 118 eukaryotic and 32 prokaryotic signal peptides, von Heijne (1985) attempted to ascertain by statistical analysis, if there were limitations to the variations that could occur within these domains. The study reemphasises the variations that do occur but also suggests that minimal requirements for the distinct regions may exist. Briefly, comparison of the sequences showed that the length of the N-region can vary considerably but that the overall net charge does not. On average, in prokaryotes anyway, the mean charge is approximately +1.7. In the hydrophobic core there are no sequence restraints and the choice of hydrophobic amino acid in this region is random. The minimum length of the H-domain is 7 - 8 residues and this region spans from positions -6 to -13 and -7 to -14 in eukaryotes and prokaryotes respectively. Extremes and exceptions do exist. Human pancreatic polypeptide and *C.diptheriae* toxin for example, have H-domains of 15 and 16 amino acids, and, the H-region of human choriogonadotrophin β-subunit signal peptide has 9 residues, 7 of which are leucine.
The cleavage site at the C-terminus is the least variable of the three. The length seldom varies from 5 amino acids in eukaryotes and 6 in prokaryotes. Also, certain sequence restraints are adhered to in that particular amino acids are preferred at particular positions in the cleavage site (section 1.3.4). These observations highlight the degeneracy of signal peptides at an amino acid level. However, they also underline the physical organisational pattern that signal sequences adhere to. The potential importance of these structural domains in maintaining signal function and the possible role of each in

the translocation process has been investigated.

(1.3.2) **Disruption of the hydrophobic domain.**

Extensive studies of signal peptide mutants have shown that disruption of the structural domains can affect leader peptide function with varying degrees of severity. *In vitro* mutagenesis of the H-domain of prokaryotic and eukaryotic signal peptides has generated mutants that significantly affect protein secretion. Hydrophobic regions of polypeptides have the potential to form α-helices, a thermodynamically preferred conformation in the non-polar environment of the lipid bilayer. It has been suggested that this region of the signal peptide interacts directly with the membrane during translocation (Wickner *et al.* 1980; Engleman, 1981). For this reason, a great deal of attention has focused on disruption mutants in the H-core of signal sequences.

Analysis of *E.coli* secretory protein mutants has shown that disruptions to the H-domain can severely affect secretion. Mutations of the lamB signal peptide, were isolated by constructing a fusion between it and β-galactosidase, a protein not normally secreted by this organism (Emr and Silhavy, 1980; Emr *et al.* 1980). Overexpression of this hybrid gene in *E.coli* results in β-galactosidase being directed to the cytoplasmic membrane, effectively jamming the normal export system. Because secretion is blocked, the cell cannot secrete its own enzymes and is prevented from using maltose as a carbon source (maltose sensitive). Mutations that alter secretion of the hybrid protein and therefore relieve jamming of the membrane result in normal secretion patterns and a maltose resistant phenotype. The mutations isolated in this manner, disrupting the function of the lamB signal peptide, all map to a group of 4 amino acids in the hydrophobic core. Some introduce a charged residue in the region while others yield various deletions. Similar results are obtained when β-galactosidase is fused to the signal peptide of another secretory protein, the maltose binding protein (MBP), in *E.coli* (Bedoulle *et al.* 1980).

In eukaryotes, the yeast secretory protein invertase has been

used for similar analyses. *S.cerevisiae* produces two types of invertase enzyme. Both are encoded by separate mRNA transcripts synthesized from a common gene, SUC2 (Perlman *et al.* 1982). The cytoplasmic form is constitutively produced and transcription of the secreted form is glucose repressed. When grown on sucrose as a carbon source, the secreted form is transcribed and the enzyme is produced in the precursor form with a signal peptide that directs the enzyme to the secretory pathway where it is transported to the periplasmic space.

Deletion of 4 amino acids in the H-region of the signal peptide, reducing it from 8 to 4 residues, resulted in accumulation of active enzyme in the cytoplasm (Perlman *et al.* 1986). Western blot analysis revealed that the mutant enzyme, now located in the cytoplasm, was not glycosylated and retained its signal peptide. This indicates that the enzyme had no interaction with the ER membrane. (Disruption of the invertase signal peptide cleavage site alone, inhibiting signal processing does not interfere with translocation (Schauer *et al.*1985).

Along a similar line, human lysozyme is efficiently secreted and processed in yeast when fused to a synthetic signal peptide constructed to optimise the H-region (Yamamoto and Kikuchi 1989). In this signal, 8 contiguous leucine residues make up the hydrophobic core. Leucine was chosen for this purpose because of its hydrophobic nature and its disposition toward forming α-helical structures (Chou and Fasman, 1978). Reduction of this core to just 6 leucine residues resulted in a decrease in secretion efficiency to 40% compared to the 8 leucine sequence.

Overall, mutant leader peptides seem to have varying effects on translocation efficiency. In bacteria, point mutations and substitutions, sometimes affecting only one amino acid can have drastic consequences blocking secretion completely. In eukaryotes however, mutations that intefere with signal function and block secretion tend to be large deletions or rearrangements (Perlman *et al.* 1986; Gething and Sambrook, 1982). In some cases, as outlined above, partial signal

function is retained. In bacteria also, deletions in the H-region of the outer membrane lipoprotein (lpp) of *E.coli* disrupt secretion to different extents, some deletions having more pronounced effects than others (Inouye *et al.* 1984). Certain mutations therefore, even in this highly conserved core retain the ability to translocate proteins, albeit with lower efficiencies.

To investigate the extent that alternate sequences can function as signal peptides, Kaiser (1987) adapted the secretion of invertase in yeast as a model test system. The invertase signal peptide was removed and replaced with random peptide sequences derived from human genomic DNA. 20% of these random sequences were shown to contain some signal function, allowing the secretion of invertase, reflected by the ability of these cells to grow on sucrose. Although the sequences secreted invertase with different efficiencies (only a small amount of secreted enzyme is required for growth on sucrose), all allowed a proportion of the enzyme to be translocated into the lumen of the ER where core glycosylation took place. Sequence comparisons to non-functional isolates showed the functional ones to be enriched in hydrophobic residues and depleted in charged amino acids.

Revertants of the non-functional, secretion defective isolates generated in this study were also examined (Preuss and Botstein, 1989). The revertants were selected by their ability to grow on sucrose as a sole carbon source after treatment with the mutagen ethylmethylsulphate (EMS), or with ultra violet (UV) irradiation. Spontaneous revertants were also isolated. Sequencing the revertant leaders showed that the mutations allowing secretion were all point mutations that either introduced hydrophobic residues or deleted charged residues in the N-terminal random sequences.

Kaisers experiments demonstrate the broad range of sequences that can function, however inefficiently, as signal peptides allowing targeting and translocation to the ER membrane. The hydrophobic domain is clearly important and the functional sequences isolated in the above studies resemble known signal

peptides in this respect. This region has a potential role in membrane targeting as well as interacting with the ER membrane itself. However, the specificity of the export process is unlikely to depend on the presence of a hydrophobic domain alone, especially considering that most proteins contain hydrophobic sequences. The inefficiency of the sequences isolated in Kaisers experiments underline this.
The conservation of overall signal peptide structure, as already mentioned, goes further than the presence of this hydrophobic domain. A positively charged amino terminus and the cleavage site region are equally well conserved (von Heijne, 1985). The roles of these domains in the translocation process and overall signal function and their interaction with each other have also been considered.

(1.3.3) **The hydrophillic domain: the role of positively charged amino acids.**

The genetic selections used to generate signal peptide mutants *in vivo*, some of which have been described, all yielded disruptions mapping to the hydrophobic domain. In no case were alterations to the hydrophillic N-domain of the signal peptide obtained that resulted in a secretion defective phenotype. The hydrophillic domain however is a conserved feature in all signal peptides and in prokaryotes, secretory proteins carry a net positive charge in this region (von Heijne 1984a).
The potential importance of these charged residues and their possible role in prokaryotic signal function, was first proposed in the "loop model" for protein translocation (Inouye and Halegoua, 1982). According to this model, the N-terminal of the signal peptide does not leave the cytoplasm. The basic amino acids in this region are proposed to interact with the acidic groups in the phospholipids of the bacterial cytoplasmic membrane, anchoring it in the cytoplasm and allowing the hydrophobic core to loop into the membrane. The electrochemical potential that exists across the bacterial cytoplasmic membrane, which is negatively charged on the cytoplasmic side, would promote this type of interaction (Li *et al*. 1988).

Oligonucleotide site-directed mutagenesis has generated mutations mapping to the hydrophillic N-domain of prokaryotic and eukaryotic signal peptides. A lot of interest has focussed on positively charged amino acids in this region. Although the N-termini of all prokaryotic signal peptides contain at least one basic amino acid, evidence suggests that this feature is not absolutely required for signal function. Removal of the 3 basic residues in the maltose binding protein (MBP) of *E.coli*, for example, has no effect on export of the protein (Puziss *et al.* 1989). Similarly, when the 2 lysine residues are removed from the prolipoprotein signal peptide, no discernable effect on translocation is observed (Vlasuk *et al.* 1983). However, a net charge of at least +1 is required to obtain correct processing when this signal peptide is fused to β-lactamase (Lunn *et al.* 1987). The introduction of net negative charges in the N-domain of prolipoprotein and MBP does affect translocation, decreasing the kinetics of the process considerably (Vlasuk *et al.* 1983; Pusiss *et al.* 1989). Translocation takes place, but at a slower rate compared to the wild type sequences.

These observations have led to the suggestion that the N-domain of the signal peptide plays a facilitative role in translocation. The presence of positive charges, while not absolutely necessary, may act to improve the efficiency of the whole process. The exact function of these charged residues however remains unclear. Although it has not been proved conclusively, cross-linking studies suggest that the SecA protein, a cytoplasmic component of the *E.coli* secretory pathway that is essential for translocation, interacts with the positive charges in the OmpA signal peptide (Akita *et al.* 1990). The extent of the interaction is dependant on the number of positive charges present in the hydrophillic domain. The SecA protein also interacts with other known secretory pathway components and could therefore mediate interactions between the signal peptide and other components involved (Fandl *et al.* 1988). PrlD2 supressor strains can partially rescue the phenotype of MBP N-domain mutants (Puziss *et al.* 1989). The

prlD2 supressor strain, which is allelic to SecA (Fikes and Bassford, 1989), has no effect on hydrophobic core mutations and it is possible that SecA interacts more strongly with the hydrophillic section of the signal peptide.

In yeast, a potential role for the hydrophillic domain of signal peptides is less easy to define. Not all yeast signal peptides exhibit a net positive charge in the N-domain (Kaiser and Botstein, 1986) and the ER membrane does not contain a general electrochemical potential. None of the random sequences generated by Kaiser (1987) that promoted translocation of invertase, contained hydrophillic segments or positive charges. In contrast to this, with a similar experiment carried out in *E.coli*, most of the functional leader peptides derived from random sequences did contain a net positive charge preceding the hydrophobic domain (Zhang and Broome-Smith, 1989).

The signal peptide of the yeast secretory protein alpha-factor, has an arginine residue at position 3 in the pre-region. The position of this positively charged residue rather than it's presence appears to be an important feature in the functioning of this leader peptide (Green *et al.* 1989). Replacement of this amino acid with a neutral phenylalanine residue, has no effect on secretion. Misplacement of the positive charge by one position however, has a significant effect, reducing translocation efficiency by up to 70%.

Green (1989) proposes that misplacement of the positive charge in the α-factor pre-region may adversely affect interactions of the protein precursor with molecular chaperones such as heat shock proteins (hsps) (section 1.4.4), or a similar interaction with other components of the secretory pathway. It has also been suggested that this charged residue may be important in intramolecular interactions with a series of 3 contiguous acidic amino acids in the mature region of the α-factor protein (at positions 7-9). Transposition of the charged residue could promote or destroy an electrostatic interaction in this region, resulting in a reduction in translocation efficiency.

The interaction of positive charges in the mature sequence of prokaryotic secretory proteins and the N-domain of signal

peptides has been investigated. A net positive charge at the extreme N-terminus of chicken triosephosphate isomerase, when fused to the *E.coli* lamB signal peptide, has a detrimental effect on translocation of the protein (Andrews *et al.* 1989; Summers and Knowles, 1989). As the positive charges are moved further away from the N-terminus, by the insertion of lamB sequences, the blocking effect weakened. The introduction of positive charges into this region of the mature alkaline phosphatase sequence results in a similar defect in translocation efficiency (Li *et al.* 1988). A study of prokaryotic signal peptides (von Heijne, 1986) shows that in most cases there is a net negative or neutral charge in the area including the C-domain and the extreme N-terminus of the mature sequences of secretory proteins. This characteristic may have evolved to avoid the detrimental effects positive charges can have on translocation of certain proteins. No such pattern is observed however, in the majority of eukaryotic secretory proteins.

The potential importance of the N-domain in the folding of secretory precursors has been mentioned. Positive charges in this region of yeast secretory proteins appears to be important in some cases only. Signal peptides, having evolved with a specific protein, will presumably be optimised for the translocation needs of that particular molecule, taking into account its charged regions, folding characteristics and conformational requirements. In many cases, the particular properties of some signal peptides or the requirements of certain proteins to achieve translocation only become apparent when specific characteristics are modified, or in heterologous fusion experiments.

It seems likely that the N-domain is important in improving the efficiency of the translocation process. With the exception of hsps, specific components that interact with this region have yet to be identified in yeast. The recent identification of proteins involved in early tranlocation events in the yeast secretory pathway (section 1.4.2) may reveal more information about protein interactions in this region.

(1.3.4) **The signal peptide cleavage site.**

The C-terminal of signal peptides, containing the cleavage site, is the only region where the conservation of amino acid sequences is observed. A statistical analysis of sequences around the cleavage site of prokaryotic and eukaryotic leader peptides shows that certain patterns of amino acids are preferred in this region. (von Heijne, 1983, Perlman and Halvorsen, 1983). These studies have revealed that small neutral amino acids predominate at positions -1 and -3 (position -1 being defined as one amino acid upstream of the actual cleavage site), and that aromatic residues are strictly avoided at these positions but are present at position -2. More extensive analyses show that alanine is the preferred residue at -1 and -3 and, that an order of preference exists for other acceptable amino acids at these positions (von Heijne, 1983).

A general model has been proposed for how signal peptides maintain their cleavage specificity (von Heijne, 1984b). This model incorporates the position of the last residue of the H-domain (occurring at position -6 in eukaryotes and -7 in prokaryotes). The end of the H-domain defines a window of potential cleavage sites that compete for access to the signal peptidase. Cleavage occurs at the most probable site defined by the observed amino acid preferences revealed in the study outlined above. Although this model is based on statistical analysis of known signal sequences, a study of approximately 300 eukaryotic, prokaryotic and viral leader peptides (Watson 1984), showed that for 74% of viral and eukaryotic proteins, cleavage occurred at the site with the highest processing probability. 11% of these proteins were cleaved at the second most likely site. For prokaryotic signal sequences, 39% of actual cleavage sites were as predicted with 15% at the second most likely site.

Analysis of signal sequence termini shows that in many cases greater than one potential cleavage site exists and *in vivo* cleavage heterogeneity does occur. Bovine growth hormone for example, is processed at an alanine residue in 65% of cases, the remaining 35% of cleavage events occurring one position

upstream (Lingappa *et al.* 1977). Human interferon when produced in yeast is processed at more than one site (Hitzeman *et al.* 1983), and cleavage of rat prolactin can be forced to take place at a less favourable site by replacing one amino acid at the ideal cleavage site (Hortin and Boime, 1981).

(1.4) Proteins that interact with signal peptides.

(1.4.1) The signal recognition particle (Srp) and Srp receptor.

The first components of the eukaryotic secretory pathway identified to be involved in sequestering secretory proteins, were the srp and srp receptor (section 1.2.2). Disassembly, fractionation and reconstitution experiments (summarized by Seigel and Walter, 1988a), have allowed the detailed characterization of mammalian srp and its functions. Srp is made up of 6 polypeptides and a 7SL RNA molecule. The complex, which is held together by protein/protein and protein/RNA interactions, is arranged into 4 units consisting of 2 dimers (p9/14 and p68/72) and 2 monomers (p19 and p54).

Srp recognizes the signal peptide of a nascent secretory protein as it emerges from the ribosome (Walter and Blobel, 1981). The specificity of this recognition and binding is dependant on nascent chain length. The efficiency of srp directed targeting drops dramatically as polypeptides emerging from the ribosome exceed approximately 140 amino acids in length (Siegel and Walter, 1988b). Translation arrest, which occurs after binding to the signal peptide and ribosome and which is a characteristic feature of membrane targeting in higher eukaryotes, is associated with the p9/14 dimer subunit of srp (Siegel and Walter, 1988a). Photocrosslinking experiments show that the 54kDa polypeptide of srp specifically binds signal peptides of various secretory proteins (Kurzchalia *et al.* 1986; Krieg *et al.* 1986). Sequence analysis of this polypeptide reveals that it is made up of 2 domains, a G-domain which has the potential to bind GTP, and an M-domain, rich in methionine residues (Romisch *et al.* 1989; Bernstein *et al.* 1989). This protein also shares homology with the srp receptor

which is present on the ER membrane.

Proteins sharing homology with parts of the mammalian srp have been identified in *E.coli* and yeast (*S.cerevisiae* and *Sz.pombe)*. In yeast, the genes were isolated on the basis of their sequence homology to the p54 subunit of mammalian srp and predict protein sequences that are 50% identical to the mammalian counterpart (Hann *et al.* 1989). The yeast proteins contain a G-domain and an M-domain and although no functions have been designated as of yet, they represent the first evidence of srp related proteins in yeast. In *E.coli*, a 4.5S RNA molecule which specifically binds a 48kDa (p48) protein has been identified (Ribes *et al.* 1990). The mammalian srp component, p54, can also bind this RNA in a complex that can functionally replace the 4.5S RNA/p48 complex to promote translocation of β-lactamase in *E.coli*. Also the mammalian 7S RNA species can functionally replace the *E.coli* 4.5S RNA. This *E.coli* 4.5S RNA and p48 are thought to be structural and functional homologues of the mammalian species and are involved in the transloocation of at least some of *E.coli*'s secretory proteins.

Srp has a close affinity for its receptor in the ER membrane. Interaction with the srp receptor results in displacement of srp from the nascent chain/ribosome complex, a process which involves GTP hydrolysis (Connolly and Gilmore 1989). This interaction results in transfer of the signal peptide to the ER membrane where it is found in close proximity to a 34kDa integral membrane protein known as the signal sequence receptor protein (ssr) (Weidmann *et al.* 1989). The ssr protein is a constituent of the protein environment where secretory proteins are transferred through the membrane. Antibodies raised against the protein inhibit translocation across microsomal membranes *in vitro* (Hartmann *et al.* 1989) and it seems likely that this protein forms part of a putative translocation complex in the ER membrane. Further investigations should reveal more information about the nature of this complex and the mechanisms by which translocation, after the targetting event, is achieved.

(1.4.2) **Proteins involved in the yeast translocation event.**

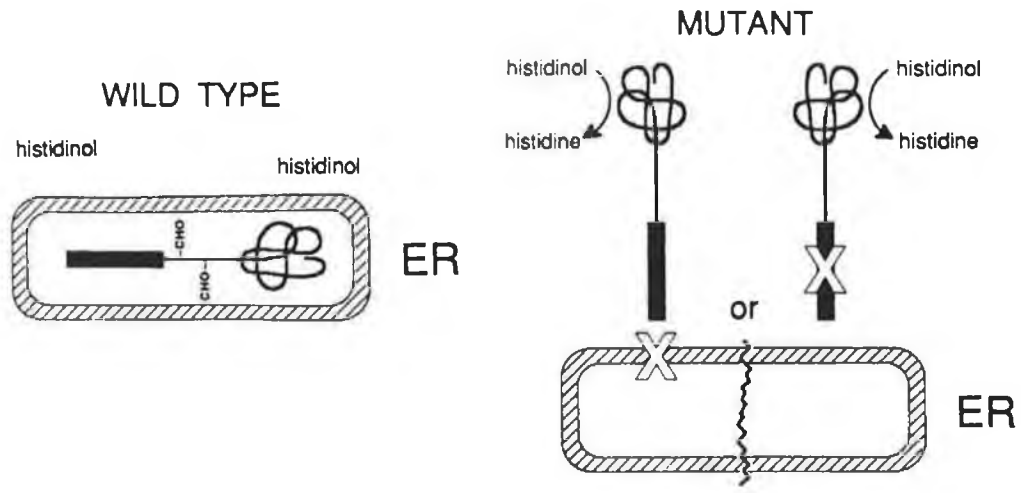
Genetic selections have been used to isolate components of the yeast secretory process (section 1.2). None of the temperature sensitive, secretion defective (*sec*) mutants isolated in these initial studies however, uncovered steps in the early translocation stage of secretion. The earliest acting mutants isolated in this screen were the glycosylation mutants affecting proteins after translocation into the ER (Ferro-Novick *et al.* 1984).

A more refined assay, developed to specifically target translocation events (described in detail in Fig.1.4), resulted in the the identification of three genes SEC61, SEC62 and SEC63 (Deshaies and Schekman, 1987; Rothblatt *et al.* 1989). *In vivo* and *in vitro* studies show that at the non-permissive temperature, these temperature sensitive mutants block transport of secretory and vacuolar proteins into the ER (Deshaies and Schekman, 1987 and 1989) and accumulation of unprocessed precursors occurs in the cytoplasm. Interestingly, all three mutants isolated in this screen encode products that are membrane associated, no yeast equivalents of srp-like components were identified, although Sec61 is a possible candidate.

Initially Sec62 was shown to be an integral membrane protein (Deshaies and Schekman, 1989 and 1990) and sequence analysis of SEC63 reveals that the gene product has a potential membrane spanning domain (Sadler *et al.* 1989). Antibodies to the Sec62 protein were used to investigate the nature of the protein's association with the ER membrane (Deshaies *et al.* 1991). Immunoprecipitations using this antibody on solubilised membranes, showed that Sec62 is associated with Sec61 and Sec63 in the ER membrane, comprising a multisubunit membrane associated complex. Furthermore, Sec61 appears to have a labile association with this putative complex, suggesting a potential intermediary role, possibly involved in targetting. Two other proteins of molecular weights 31.5 and 28kDa were precipitated along with this complex. Based on molecular weight comparisons, these proteins were tentatively proposed to be comparable to 2 subunits of the mammalian ssr protein. It is likely that this

complex is involved in the actual translocation of secretory proteins at this site in the ER membrane. Further manipulations, including reconstitution experiments, may uncover the mechanism by which proteins are translocated across membrane structures in yeast, the details of which have proved elusive in the less easily manipulated mammalian systems.

Figure (1.4) Selection of translocation mutants in yeast (taken from Deshaies *et al*, 1989).



The His4 protein (His4p) converts histidinol to histidine and is normally a cytoplasmic protein. When modified with a signal peptide, His4p is targeted to the ER. Cells expressing this fusion fail to grow on minimal medium supplemented with histidinol, due to the inability of the substrate to gain access to the ER. By selecting for mutants that could grow on histidinol, temperature sensitive mutants that disrupted translocation of His4p to the ER were isolated.

(1.4.3) The importance of conformation for translocation competence.

A series of cytosolic proteins have emerged as essential components of the eukaryotic and prokaryotic secretory process, interacting with secretory precursors to promote translocation across the ER and cytoplasmic membranes. Proteins from bacterial, mammalian and yeast cells have been identified that are involved in binding secretory precursors to promote and maintain an unfolded, translocation competent state. These proteins are termed chaperones.

Evidence for the unfolded state was first shown for the import of mitochondrial precursors (Eliers and Schatz, 1986). Pre-secretory proteins in bacterial and eukaryotic cells also exhibit similar conformational requirements for translocation. In *E.coli*, evidence for the unfolded state comes from the examination of pre-secretory forms of OmpA and MBP (Crooke and Wickner, 1987; Randall and Hardy, 1986). Both of these proteins are sensitive to protease digestion in their presecretory forms only, suggesting a loosely folded conformation. In some cases, artificial denaturation, using urea, can render pre-proteins competent for translocation *in vitro* without the addition of cytosolic extracts (Sanz and Meyer, 1988). Dilution of the urea results in renaturation of the protein and is accompanied by a loss of translocation competence.

In cells where translocation has been artificially blocked, pre-MBP remains in a loosely formed conformation for up to 10 minutes (Randall and Hardy, 1986). The factor involved in maintaining the unfolded conformation of pre-MBP is the SecB protein (Collier *et al.* 1988). SecB$^-$ strains exhibit a severe defect in export of MBP, and pre-MBP isolated from *secB*$^-$ strains was shown to be in a tightly folded, protease resistant form (Kumamoto *et al.* 1988). Purified SecB can prevent the formation of protease resistant pre-MBP *in vitro* and can also block the refolding of artificially denatured pre-MBP (Collier *et al.* 1988; Liu *et al.* 1988). SecB can also maintain proOmpA in a translocation competent state *in vitro* (Lecker *et al.* 1989). *In vivo* however, *secB*$^-$ strains are not defective in OmpA

export and *secB*⁻ cell extracts can still promote OmpA translocation *in vitro* (Kumamoto *et al.* 1989). Another chaperone protein, trigger factor, has been shown to specifically interact with proOmpA to promote translocation in much the same way as SecB (Crooke and Wickner 1987). This evidence suggests that there are several chaperone proteins involved in protein export and that different ones are specific for different proteins.

Eukaryotic cells also appear to require the presence of molecular chaperones and it has been suggested that srp may act as such in mammalian cells. Srp can replace trigger factor to stabilise proOmpA for translocation across bacterial, yeast and mammalian membrane structures (Sanz and Meyer, 1988). It is also functional in promoting prepro α-factor translocation into yeast microsomes (Crooke *et al.* 1988). Denaturation of these proteins prior to the addition of srp was required to allow translocation. This correlates with the co-translational targeting role of srp *in vivo* and the general idea that chaperones are likely to bind precursors as they are synthesized rather than actively unfolding the completed proteins.

(1.4.4) **Unfolding in yeast: the role of heat shock proteins.**

In yeast, genetic and biochemical evidence suggests that members of the heat shock family of proteins (hsps) play a role in promoting translocation competence in pre-secretory proteins (Chirico *et al.* 1988). When prepro-α-factor is produced in wheat germ lysate, it can only be translocated into yeast microsomes if yeast cytosolic extract is added. Fractionation of the extract showed that this activity is associated with 2 yeast hsps, SSA1 and SSA2 (members of the 70kDa hsp family). The effect can also be achieved if the protein is denatured before addition of the yeast membranes. This reinforces the idea that proteins must be unfolded in order to be translocated in yeast. The involvement of these proteins is backed up at a genetic level in that *ssa1* mutants accumulate α-factor, carboxypeptidase-Y and mitachondrial precursors in unprocessed,

untranslocated forms (Werner-Washburner *et al.* 1987).
The *in vitro* evidence involving fully formed α-factor, suggests that hsps can act in a post-translational capacity. It has been proposed that these proteins may act as "unfoldases", possibly using energy derived from ATP hydrolysis to actively unfold protein precursors before translocation (Rothman and Kornberg, 1986). Hsps have also been implicated in post-translational translocation in mammalian cells (Zimmerman *et al.* 1988) and in *E.coli* where the GroEL protein has been shown to interact with β-lactamase to promote its translocation (Kusukawa *et al.* 1989).

(1.4.5) **Characterization of signal peptidases.**
Two *E.coli* signal cleavage enzymes have been identified, leader peptidase or SP1 (Zwizinski and Wickner 1980) and lipoprotein peptidase or SP11 (Tokunaga *et al.* 1982). Both signal peptidases are integral membrane proteins with molecular masses of 36 and 18kDa respectively. Repression of leader peptidase function results in accumulation of unprocessed precursors in the *E.coli* periplasm (Dalbey and Wickner, 1985) This suggests that in *E.coli*, while signal peptidase is not essential for translocation, retention of the signal peptide inhibits the release of secretory proteins into the periplasm.
Signal peptidases of higher eukaryotic cells appear to be more complex. Canine and avian signal peptidases have been isolated as multisubunit complexes (Evans *et al.* 1986; Baker and Lively, 1987). Canine microsomal signal peptidase consists of 6 polypeptides and hen oviduct signal peptidase exists as a heterodimer. Two of the canine and one of the avian subunits are glycosylated and share sequence homologies (Shelness *et al.* 1988). Despite the differences in complexities, the substrate specificities of prokaryotic and eukaryotic signal peptidases are remarkably similar and both enzymes cleave eukaryotic and prokaryotic signal peptides at the correct cleavage sites (Watts *et al.* 1983; Muller *et al.* 1982). The extra subunits of the vertebrate enzyme complex may provide additional functions associated with increasing the efficiency or cleavage

specificity of the signal cleavage process in vertebrates (Randall and Hardy, 1989).

Disruption of signal peptide cleavage sites in yeast secretory proteins results in retention of the signal peptide. Translocation of the mutant protein is not affected. Replacement of an alanine residue with valine at position -1 in the secreted form of invertase inhibits cleavage and the unprocessed form of the enzyme is translocated into the lumen of the ER (Schauer *et al.* 1985). Although translocation is achieved, the mutant invertase accumulates in the ER and further secretion is delayed. This has been shown to be a result of insolubility of this form of invertase in the lumen of the organelle, combined with a possible association between the mutant protein and the ER membrane *via* the signal peptide (Schauer *et al.* 1985). Another yeast secretory protein, acid phosphatase, is also transiently retained in the ER when signal cleavage is inhibited (Haguenauer-Tsapis *et al.* 1986).

A mutation to the yeast signal peptidase would presumably mimic this phenotype of delayed secretion patterns. One of the yeast temperature sensitive *sec* mutants, *sec*11, was shown to transport invertase and acid phosphatase at severely reduced rates (Bohni *et al.* 1988). At the restrictive temperature, *sec*11 cells accumulate core glycosylated acid phosphatase and invertase with their signal peptides still intact. The SEC11 gene was cloned and shown to encode an 18.8kDa integral membrane protein with one potential glycosylation site. The mass of the protein is very similar to the avian and canine glycosylated subunits described and although it does not share exact sequence homologies with the canine subunit, the two proteins have similar arrangements of hydrophillic and hydrophobic domains (Shelness *et al.* 1988). The SEC11 gene product is essential for signal peptidase activity in yeast. At this stage it is not clear if the yeast signal peptidase, as it is now referred to, acts as part of a complex. Also it is not known if the Sec11 protein is responsible for the actual cleavage event. A method has been developed for the solubilisation of microsomal membranes retaining signal

peptidase activity and should allow further characterisation of the Secl1 protein (Ya Deau and Blobel, 1989).

(1.5.0) **Sorting signals in the secretory pathway.**

In addition to secretory proteins, the ER contains proteins bound for membrane structures, proteins en route to the Golgi and proteins destined to remain in the ER itself. A large number of these ER proteins interact with incoming proteins allowing them to fold and mature before being passed on (Pelham 1989). An intriguing question which arises out of studying this transport process, is how the cell sorts these proteins. What distinguishes ER resident proteins from those that are to be passed on and how are proteins targeted to their final destinations?. Also, in the Golgi, how are proteins destined for vacuolar compartments diverted from the bulk flow of protein traffic into vacuole specific vesicles? While the question of protein sorting in the secretory pathway of eukaryotic cells is far from fully understood, the investigation of two sorting events has allowed some insights into the mechanisms involved and the types of signals used.

(1.5.1) **Retention in the ER.**

Proteins enter the ER in an unfolded state (section 1.4). In order to be transported further, refolding, assembly and ER modifications must first take place. In mammalian cells, an ER protein known as BiP (binding protein) was found associated with malfolded secretory proteins that were blocked from further transport in the ER of mammalian cells (Gething *et al*, 1986). Enhanced expression of the protein was observed under conditions that promote the accumulation of unfolded or mutant proteins (Kozutsumi *et al*, 1986). BiP was also found to be transiently associated with wild type forms proteins in the ER (Bole *et al*, 1986). It was proposed that BiP, and possibly other ER proteins, could be involved in monitoring the folding of secretory proteins, allowing correct assembly, folding and modifications to take place before further transport

(Normington *et al* 1989).The yeast homologue of BiP acts in a similar fashion to its mammalian counterpart (Rose *et al* 1989; Normington *et al* 1989). Expression of yeast BiP is induced when secretory proteins accumulate in the ER, such as in *seec*53 mutants or after treatment with tunicamycin (section 1.6).

The identification of BiP and other ER resident proteins such as PDI (protein disulphide isomerase), has allowed some insight into ER sorting mechanisms. Comparison of BiP and PDI sequences reveals a common carboxyl terminus of four amino acids, KDEL (Munro and Pelham, 1987). Removal of this KDEL sequence results in secretion of the proteins (Mazzarella *et al* 1990). The addition of the tetrapeptide to the C-terminal of secretory proteins, results in their accumulation in the ER (Pelham, 1988). Experiments with *S.cerevisiae* BiP and PDI proteins have yielded similar results, although in this organism the retention signal differs to the mammalian by one amino acid ie. HDEL instead of KDEL (Hardwick *et al* 1990). In *Sz.pombe*, the sequence is ADEL while *K.lactis* recognizes both DDEL and HDEL (Inhohara *et al*, 1989; Lewis *et al*, 1990).

In *S.cerevisiae*, secretory proteins tagged with HDEL accumulate in the ER, having acquired modifications known to occur in early Golgi compartments (Dean and Pelham, 1990) (see section 1.6 for glycosylation of secretory proteins). This observation led to a model for retention based on retrieval. It was proposed that ER proteins can leave the ER in secretory vesicles but are retrieved by interaction with a putative membrane bound receptor. This receptor would bind the HDEL sequence and return the proteins to the ER *via* a special set of transport vesicles.

This receptor (the ERD2 gene product) was identified in yeast by the isolation of mutants that failed to retain secretory proteins tagged with the HDEL retention sequence (Semenza *et al* 1990). The gene was shown to encode a membrane spanning protein, the expression levels of which control the capacity of the retention system. This gene was also cloned from *K.lactis*. (Lewis *et al* 1990). *S.cerevisiae*, carrying the *K.lactis* ERD2 gene, recognises both HDEL and DDEL sequences, demonstrating

that this receptor specifically recognizes the retention signal. The human homologue of ERD2 has also been identified and the gene product localised to a post-ER Golgi-like compartment (Lewis and Pelham, 1990). While the available evidence supports a model of retrieval for ER retention in higher and lower eukaryotes, the exact nature of the interactions between signal and receptor and the process of retrieval are not fully understood.

(1.5.2) **Vacuolar sorting signals.**

The second major sorting step that has attracted much investigation is the diversion of proteins from the Golgi into secretory vesicles bound for the lysosome. In mammalian cells, tagging of acid hydrolyses with mannose-6-phosphate (Man-6-P) has been shown to signal targeting of these proteins (Sly and Fischer,1982).

The yeast equivalent of the lysosome is the vacuole, an acidic compartment containing numerous hydrolytic enzymes. Studies on proteins directed to the vacuole has led to an understanding of some of the requirements for targeting in *S.cerevisiae*. Carboxypeptidase-Y (CpY) is the best studied of these vacuolar hydrolases. It is produced as an inactive precursor (proCpY) and undergoes glycosylation in the ER before sorting occurs at the level of the Golgi (Hasilik and Tanner, 1978; Stevens *et al* 1982). In the vacuole, CpY is converted to its mature active form by the action of another vacuolar protease proteinase A (PrA), the product of the PEP4 gene (Ammerer *et al* 1986). Glycosylation is not involved in protein sorting to the vacuole in yeast and inhibition of glycosylation has no effect on localisation of CpY (Schwaiger *et al* 1982).

Fusion experiments involving portions of the proCpY N-terminal and yeast invertase, showed that the propeptide of CpY can direct invertase to the vacuole (Bankaitis *et al* 1986). This fusion protein, when expressed on a low copy number plasmid, targeted invertase to the vacuole with < 2% being secreted to the cell surface. Increasing the copy number resulted in up to 20% of the invertase being secreted, suggesting that the

targeting system is saturable. This is also the case when wild type CpY is overexpressed in *S.cerevisiae* (Stevens *et al* 1986). Deletion studies and site directed mutagenesis of CpY identified four contiguous amino acids (QPRL), at positions 24 -27 in the pro-region of the signal sequence, to be responsible for targeting of CpY to the vacuole (Valls *et al* 1987). Similar analyses with the other vacuolar hydrolase proteinase A (PrA), demonstrated that the pro region of this peptide can also target CpY to the vacuole (Klionski *et al* 1988). Sequence analysis of PrA, however, did not reveal a QPRL motif or any other similarities to CpY in this region, suggesting that greater than one recognition signal may exist.

The CpY-invertase fusion was used to isolate potential receptors involved in the targeting process. In a *suc*$^-$ strain, mutants missorting the CpY-invertase fusion to the cell surface were isolated on the basis of their ability to grow on sucrose as a sole carbon source. A large number of mutants, defining 47 complementation groups, were identified using this screen (Bankaitis *et al* 1986; Banta *et al* 1988). One mutant in particular, *vps*15 (producing a mutant protein kinase) has been analysed in detail. Study of this enzyme, which exhibits phosphorylating and autophosphorylating activities, has led to the suggestion that phosphorylation may play a role in the regulation of vacuolar recognition and sorting events in *S.cerevisiae* (Herman *et al*, 1991).

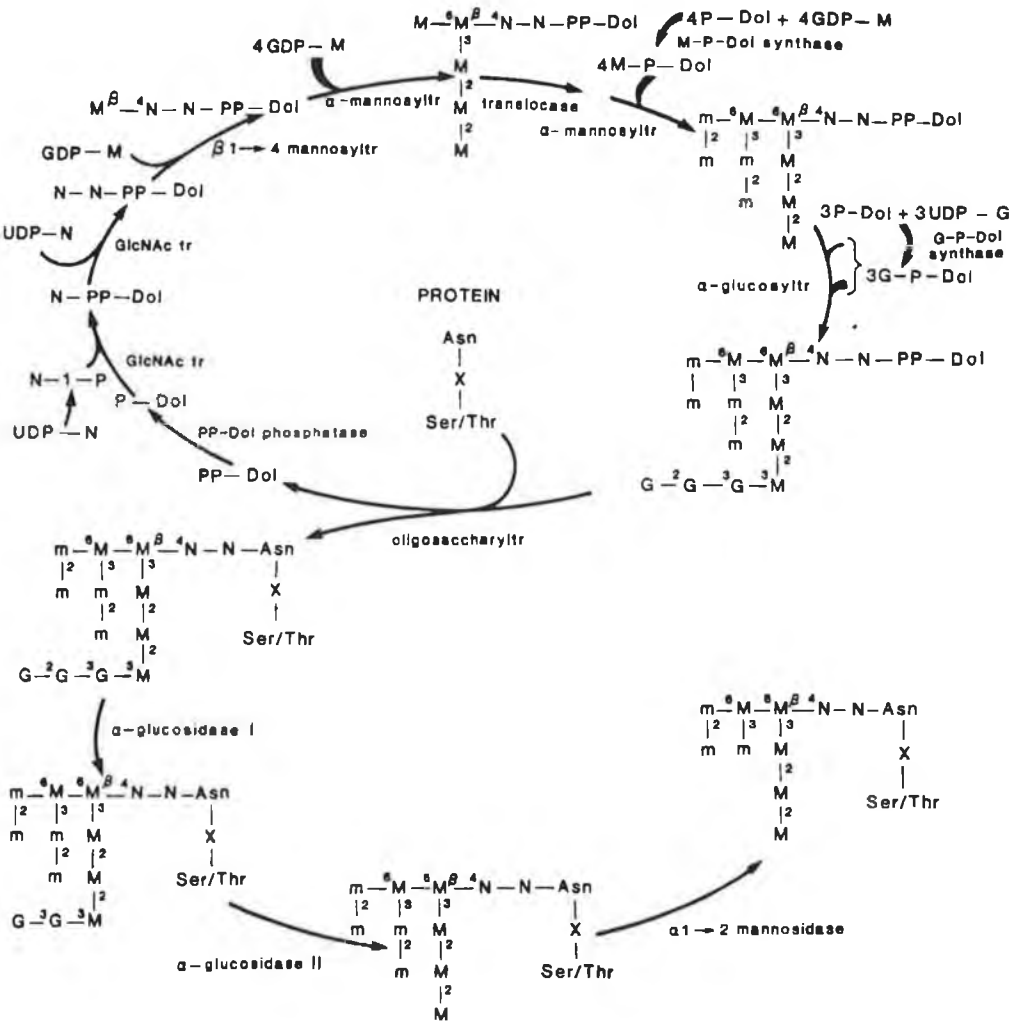
(1.6.0) **N-linked glycosylation of eukaryotic proteins.**

Secretory proteins passing through the ER membrane, acquire asparagine-linked (N-linked) core oligisaccharide chains which are subsequently modified and extended in the Golgi. Studies in yeast using mutants defective in secretion and glycosylation have proved invaluable in characterising the individual steps involved in assembly, transfer and modification of these oligosaccharides.

The assembly of oligosaccharide chains onto yeast and mammalian glycoproteins involves a large number of transfer reactions. This core oligosaccharide is assembled in both cases on a phosphorylated dolichol glycolipid (P-Dol), in the ER membrane. The core unit is made up of 14 sugars (3 glucose, 9 mannose and 2 N-acetylglucosamine residues), $Glc_3Man_9GlcNAc_2$, which are transferred to the polypeptide chain *via* an N-glycosidic bond to asparagine (Asn) residues. In both yeast and mammalian cells, this Asn residue is a substrate when it occurs within the sequence Asn-X-Ser/Thr, where X can be any amino acid except proline (Lehle and Bause, 1984). The pathway for assembly and transfer of this core oligosaccharide is similar in both systems. The differences lie in the trimming and addition reactions that are initiated in the ER and completed in the Golgi.

Much of the initial information about core glycosylation has come from investigations in mammalian cells. Yeast however, has confirmed this model with the isolation of *alg* (asparagine linked glycosylation) mutants that have allowed further characterisation of the individual steps. The assembly, transfer and trimming of the yeast oligosaccharide core is summarized below in sections (1.6.1) and (1.6.2) and in Figure (1.5) (Deshaies *et al* 1989).

Figure (1.5) Assembly of the core oligosaccharide on the ER membrane, transfer onto the protein and trimming of the core. (Taken from Deshaies *et al*, 1989)



N = GlcNAc (N-acetyl glucosamine) M = Man (Mannose)

G = Glc (Glucose) tr = transferase

Monosaccharide donors are produced by the following reactions:

Glc-6-P	→	Glc-1-P	→	UDP-Glc
Fru–6-P	→	GlcNAc-1-P	→	UDP-GlcNAc
Man-6-P	→	Man-1-P	→	GDP-Man

(1.6.1) **Core oligosaccharide assembly.**

The first step in the pathway involves the addition of GlcNAc-1-P from UDP-GlcNAc to P-Dol on the ER membrane. This initial step is inhibited by the antibiotic tunicamycin (Lehle and Tanner, 1976). Prolonged incubation of yeast cells in tunicamycin is lethal and the transferase gene (ALG7), responsible for the addition of this first GlcNAc residue was isolated from a genomic library on the basis of its tunicamycin resistant phenotype (Rine *et al* 1983; Barnes *et al* 1984). The second GlcNAc is then added to the growing oligosaccharide chain before transfer of the first mannose residue. This first mannose residue is transferred from GDP-mannose by the action of β-1,4 mannosyltransferase, encoded by ALG1. This gene and the other ALG genes, with the exception of ALG7, were isolated by their ability to complement *alg* mutants. These mutants were generated using a mannose suicide selection screen whereby glycosylation mutants survive due to their reduced ability to incorporate ^{3}H-mannose (Huffaker *et al* 1982).

The *alg*4 mutant was shown to accumulate a spectrum of oligosaccharide intermediates containing 1 to 8 mannose residues (Huffaker and Robbins, 1983). Due to the broad range of incomplete oligosaccharide units linked to the dolichol carrier observed in this mutant, it seemed likely that the ALG4 gene product would play a more indirect role in mannose addition than for example a transferase. The *sec*53 temperature sensitive mutant, defective in secretion and glycosylation of invertase and α-factor glycoproteins, was shown to be allelic to *alg*4 (Ferro-Novick *et al* 1984; Feldman *et al* 1987). Detailed analysis of the *sec*53 mutant at permissive (25^{0}C) and non-permissive (37^{0}C) temperatures in cell free translocation and glycosylation systems, has allowed characterization of the *sec*53 defect (Kepes and Schekman, 1988). Extracts and membranes from *sec*53 cells were shown to accumulate α-factor precursors with 0,1,2 and 3 core oligosaccharide units. Rescue of this phenotype and full glycosylation of the α-factor protein could be stimulated by the addition of wild type cell extract. Enhanced stimulation was observed using the cell extract of

these cells carrying the SEC53 gene on a multicopy plasmid. Supplementing *sec*53 membranes with GDP-mannose also stimulated glycosylation and it was proposed that the mutant was defective in the phosphomannomutase activity responsible for converting Man-6-P to Man-1-P *in vivo*. This was confirmed by examination of the SEC53 gene product in a surrogate bacterial host (*S.typhimurium*) where the host and yeast phosphomannomutase activities could be distinguished (Kepes and Schekman, 1988).

After the addition of the first β1-4,mannose residue, four additional mannose sugars are added in various linkages (α-1,6; α-1,3; and α-1,2), from GDP-mannose donors (see Fig.1.5). Two mutants, *alg*2 and *sec*59 have been implicated in effecting this step (Huffaker and Robbins, 1983; Bernstein *et al* 1989). *alg*2 mutants accumulate incomplete oligosaccharides containing 1 or 2 mannose residues and are temperature sensitive for growth. *sec*59 also produces a discrete set of incomplete core units ranging from 5 to 6 mannose additions. Accumulated invertase and α-factor isolated from *sec*59 cells at the restrictive temperature, contain incomplete core units that are sensitive to cleavage by endoglycosidase H (EndoH). This is unusual as the normal Man_5 intermediate is EndoH resistant, as are the *alg*3 intermediates which accumulate at the next stage of assembly (see below). GDP-mannose and Dol-P are not limiting in *sec*59 cell extracts when tested in an *in vitro* translocation / glycosylation system and it has been suggested that SEC59 may encode a mannosyltransferase (Huffaker and Robbins 1983; Bernstein *et al* 1989).

These initial steps in core unit assembly are believed to take place on the cytoplasmic side of the ER membrane. Evidence for translocation of the lipid linked oligosaccharide to the luminal face of the ER comes from experiments in mammalian cells (Snider and Rogers, 1984). Fibroblasts, treated with concanavalin A show that the oligosaccharides carrying 1 to 6 mannose sugars are on the cytoplasmic face while those with more than 5 additions are oriented toward the lumen.

*Alg*3 mutants define the next step in core assembly. $Man_5GlcNAc_2$-PP-Dol assembled oligosaccharides accumulate in

this mutant and the incomplete precursors are transferred to secretory proteins in the lumen of the ER (Huffaker and Robbins, 1983). Membranes from *alg*3 mutants synthesize Dol-P-Man, the sugar donor for the transfer of the final 4 mannose units and it is likely that this mutant is also defective in the transfer reaction.

The final step in assembly is the addition of 3 glucose residues to the core. ALG6 encodes the glucosyltransferase responsible for the addition of the first α-1,3 linked glucose while the ALG8 gene product transfers the final two (Runge and Robbins, 1986). In both mutants, transfer of underglucosylated core units to the secretory peptides still occurs. The ALG5 gene has also been defined in its role at this stage of assembly (Runge *et al* 1984). *alg*5 mutants are defective in synthesis of the Glc-P-Dol donor and affects oligosaccharide assembly indirectly, in much the same way as *sec*53 (*alg*4). Membranes from *alg*5 cells synthesize complete $Glc_3Man_5GlcNAc_2$ chains only when Glc-P-Dol is added exogenously.

(1.6.2) **Transfer and trimming of the core oligosaccharide.**

The completed core is transferred to the polypeptide in the lumen of the ER by an oligosaccharide transferase that has been identified in both mammalian and yeast cells (Kaplan *et al* 1987; Sharma *et al* 1981). Under glucosylated donor core units can also be transferred to secretory polypeptides although less efficiently than fully glucosylated units. Even severely truncated donors can be transferred, as has been discussed above with some of the *alg* mutations.

After transfer, extensive trimming reactions in mammalian cells leaves only 3 mannose residues in the core unit. This is subsequently extended by the addition of sialic acid, galactose or N-acetylglucosamine (Kornfield and Kornfield, 1985). In yeast α-glucosidase I (encoded by the GLSI1 gene) and α-glucosidase II are responsible for the sequential removal of the 3 glucose residues and an α-1,2 mannosidase removes the mannose residues (Byrd *et al* 1982). Analysis in *sec*18 strains show that these modifications occur in the lumen of the ER,

before transport to the Golgi (Esmon *et al* 1984). (*sec*18 mutants accumulate secretory proteins in the ER due to disruptions in vesicle fusion events between the ER and Golgi apparatus (Eakle *et al* 1988)). Secretory proteins accumulating at the restrictive temperature in this mutant are predominantly glycosylated with oligosaccharides containing 8 mannose and no glucose residues. Also, in a *gls1sec*18 double mutant, the accumulation of $Glc_3Man_8GlcNAc_2$ shows that removal of the two types of sugars are independent.

In contrast to mammalian glycoproteins, yeast glycoproteins are extensively modified by large numbers of mannose residues. These outer chain addition reactions occur after transfer of proteins to the Golgi (Esmon *et al* 1981). The removal of the glucose sugars is not necessary for outer chain addition and elongation although removal of the α-1,2 mannose may be (Tsai *et al* 1984).

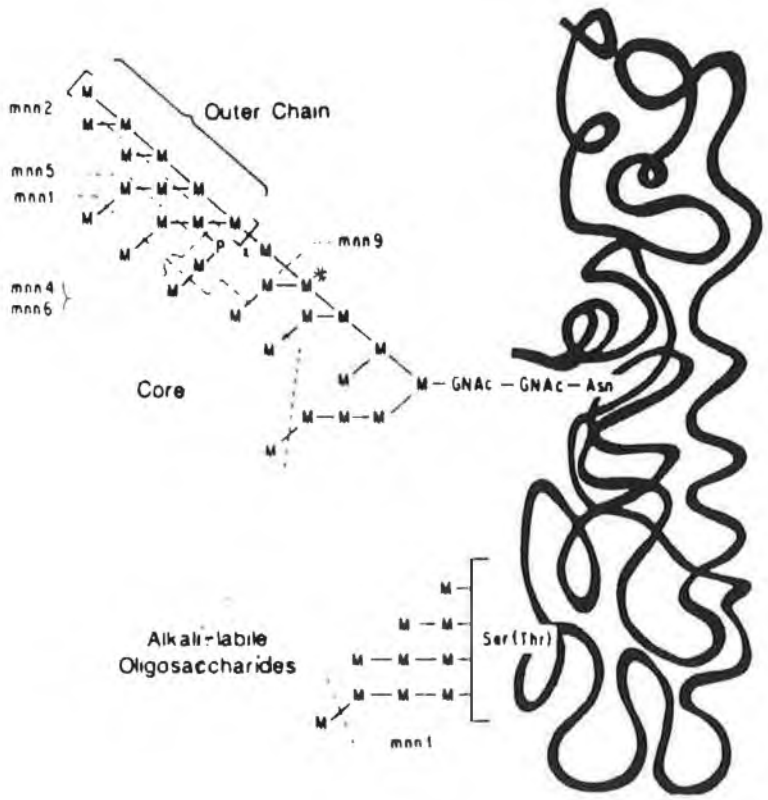
(1.6.3) **Elongation in the Golgi.**

Elongation of the core unit has been characterized using mutants defective in the addition of outer chain mannose residues. The cell wall of *S.cerevisiae* is made up of mannoproteins that are characterized by the presence of α-1,3 linked mannose units. α-1,3 mannose sugars are highly immunogenic and cells mixed with antiserum raised against these sugars cause the cells to agglutinate. The *mnn* (mannan defective) mutants were isolated on the basis of their inability to agglutinate due to defects in the cell surface glycoproteins (Ballou *et al* 1973; Ballou and Raschke, 1974). Although the mutants were isolated as cell wall glycan mutants, the disruptions affect all proteins in the N-linked glycosylation system.

Initial steps in the addition of mannose extensions were elucidated by analysis of modifications to secreted invertase (Trimble and Atkinson, 1986). Outer chain addition is initiated by the transfer of an α-1,6 mannose to the α-1,6 backbone chain of the core by an unidentified α-1,6 mannosyltransferase. Elongation occurs when a different α-1,6 mannosyltransferase

extends the core by the addition of outer chains to this initiation residue (Fig.1.6). This step is defined by the *mnn*9 mutation which lacks this specific mannosyltransferase (Tsai *et al* 1984). In wild type cells, glycoproteins can have up to 15 of these outer chains extending from the N-linked core. Side chain additions, defined by other *mnn* mutants are also indicated in Fig.(1.6). *mnn*9 mutants are less viable than both wild type and outer chain mutant strains, suggesting that outer chain synthesis is essential for cell growth.

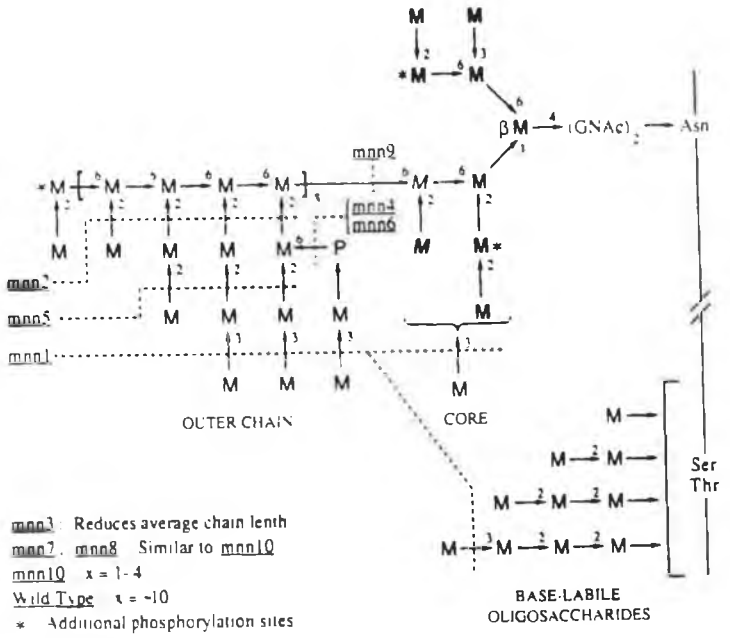
Figure (1.6) Extension of inner core oligisaccharide with mannose side chains (the initiation mannose added to the α,1-6 backbone chain is marked with an asterix). (from Kurkuruzinska *et al*, 1987)



x = 1-15 (number of outer cores that can be added).

More recent analysis of the structure of mannose additions in *mnn*9 mutants, suggests that the elongation reaction may stem from a different point in the core than previously suggested for invertase (Hernandez *et al* 1989). The discovery that the α-1,6 mannose residue previously proposed to be the initiation point for elongation is phosphorylated in *mnn*9 glycoproteins, precludes it as the point of the first mannose addition and therefore for elongation. An alternative structure is proposed in Fig.(1.7), suggesting that elongation extends from the α-1,3 mannose as indicated. While the possibility remains that different structures may occur in different glycoproteins, this proposal is more conducive with the observation that initiation and elongation are two separate events and are promoted by the action of two distinct mannosyltransferases with different specificities.

Figure (1.7). Outer chain addition extends from the α,1-3 mannose in *mnn*9 glycoproteins (the invertase α,1-6 mannose residue now phosphorylated is indicated with asterix). (Hernandez *et al*. 1989).



(1.6.4) **Glycosylation of yeast secretory proteins.**

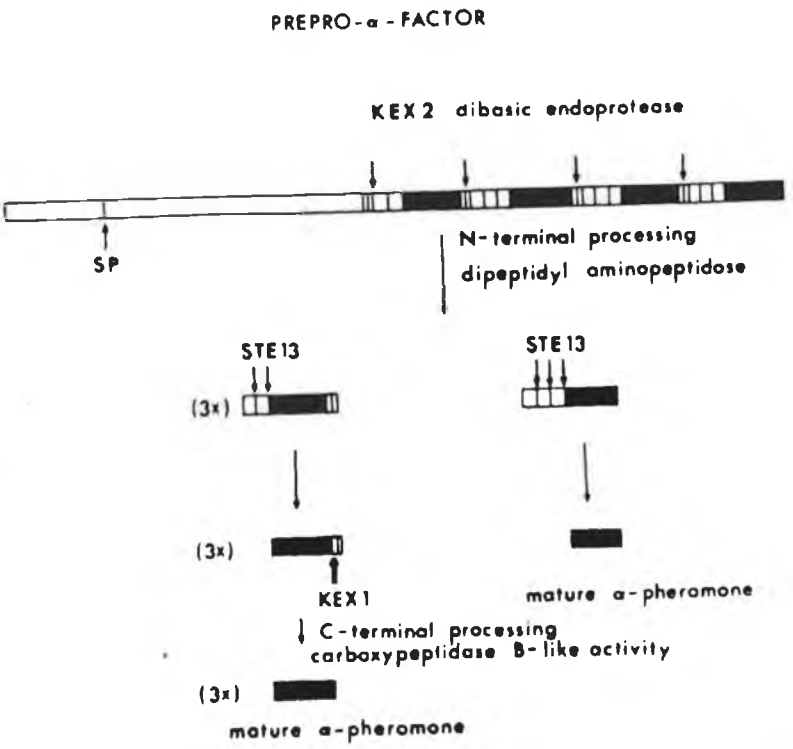
Much of the information on the yeast glycosylation process was obtained by studying glycosylation patterns of proteins naturally secreted by *S.cerevisie*. These studies have allowed some insight into the role of glycosylation in the secretion and maturation of proteins through the secretory pathway. Invertase for example, accumulates as an inactive, ER membrane bound precursor at the restrictive temperature in *sec*53 and *sec*59 cells (Ferro-Novick *et al* 1984). On return to the permissive temperature, the protein appears to refold, becomes partially active again and is secreted. The majority of secreted invertase exists as an octamer (Esmon *et al* 1987). Assembly of this octamer and its localization in the yeast periplasmic space is related to glycosylation of the enzyme. Octamers are not formed when invertase is synthesized in the presence of tunicamycin and secretion of the enzyme is inhibited under these conditions. Small amounts of invertase dimers are released from the cell wall when cells are washed with buffer or β-mercaptoethanol. Release of the octameric form of the enzyme is only achieved with cell wall disruption. This has also been shown for other periplasmic proteins such as acid phosphatase and α-galactosidase.

Studies on α-factor production in yeast has also been characterized in glycosylation defective mutants. α-factor production involves a series of complex processing reactions which are summarized in Figure (1.8) (Bussey, 1988). Three copies of the α-factor protein are synthesized as a larger precursor of 165 amino acids, 85 of which comprise the signal sequence (Julius *et al* 1983). The first 20 residues (the pre-region) are removed by signal peptidase on translocation into the ER, leaving the three copies of α-factor attached to the 65 amino acid pro-region. This pro-region has 3 glycosylation sites at positions 23, 57 and 67. Studies in *sec*18 mutants show that this region is core glycosylated in the ER. Removal of the pro-peptide occurs in the Golgi, by the action of a specific peptidase, KEX2 (Julius *et al* 1984a). Further processing requires the action of two additional

peptidases, the STE13 and KEX1 gene products (see Fig.1.8 for details)

Secretion of α-factor is blocked in *sec*59 and *sec*53 mutants at the non-permissive temperature. Incomplete and unglycosylated forms of the protein precursor accumulate in the ER, correlating glycosylation with secretion efficiency. The effect of blocking secretion with tunicamycin is more pronounced at 30^0C and 37^0C, compared to 25^0C, suggesting that inhibition of secretion may be related to folding of the precursor protein (Julius *et al* 1984b).

Figure (1.8) Processing of prepro-α-factor (Bussey, 1988).



(1.7.0) **The secretion of heterologous proteins in *S.cerevisiae*.**
The first proteins to be secreted in yeast, human interferon α-1 and interferon α-2, were directed by their natural signal peptides (Hitzeman *et al*, 1983). These heterologous signal peptides however, were processed by signal peptidase at greater than one site and heterogeneous products were recovered from the medium. Human serum albumin (HSA) on the other hand, also secreted using its own signal peptide, is correctly processed by the KEX2 cleavage enzyme when produced in yeast (Etcheverry *et al*,1986; Bathurst *et al*, 1987). This human signal peptide is surprisingly efficient, more efficient than many homologous secretion signals (Sleep *et al*, 1990) and has also been used to secrete other heterologous proteins.
As well as its use as a signal peptide in yeast, the HSA protein, with its signal sequence still intact, has been used in fusion studies. Secretion and glycosylation of invertase was achieved with the HSA protein and signal peptide fused to its 3' end. Stomatostatin has also been produced in yeast when fused to HSA (Hitzeman *et al*, 1990). These experiments demonstrate the potential of using heterologous signals, not only to secrete heterologous proteins, but also (by fusing to the heterologous protein itself) for stabilizing and secreting smaller proteins in yeast. Other heterologous signal peptides have been shown to direct their natural products through the yeast secretion system with varied efficiencies. Human and wheat α-amylases are secreted using their native signal sequences, although at a lower efficiency compared to HSA (Nakamura *et al*, 1986). α1 antitrypsin on the other hand, cannot be secreted using its native signal peptide (Rothstein *et al*, 1987).
Homologous yeast signal sequences from yeast secretory proteins, fused to the mature portions of heterologous genes are widely used to direct secretion. The invertase signal sequence efficiently directs secretion of HSA, prochymosin and interferon α2, among others (Etcheverry *et al*, 1986; Smith *et al*, 1985; Chang *et al*, 1986). The α-factor signal peptide is also widely used in the production of heterologous proteins.

The α-factor prepro-region was shown to secrete IFNα1 more efficiently than the IFN signal peptide (Singh *et al*, 1984). However, in this case the Glu-Ala repeats that occur after the Lys-Arg KEX2 cleavage site were retained in a large proportion of the secreted material. Attempts to produce this protein by direct fusion to the Lys-Arg site, resulted in inefficient cleavage by KEX2 (Zsebo *et al*, 1986) showing that heterologous sequences in the fusions can affect cleavage specificity in homologous signal peptides in some cases. Direct fusion to the Lys-Arg site of the α-factor prepro-region however, has resulted in efficient processing and secretion in most other cases (Ernst *et al*, 1986; Guisez *et al*,1990 Loison *et al*, 1988; Shaw *et al*, 1988 are examples).

A comparison of a number of signal sequences and their ability to secrete HSA has been carried out (Sleep *et al*, 1990). In this study the HSA signal, the α-factor signal and the killer toxin signal from *K.lactis* (Stark and Boyd, 1986) were compared along with two fusion signal peptides. These fusions comprised the HSA signal with 5 amino acids around the cleavage site replaced by the KEX2 cleavage site of α-factor and the killer toxin signal with its last 13 amino acids replaced by the same α-factor sequence (Sleep *et al*, 1990). Results show that both fusion peptides were more efficient at secreting HSA than any of the other 3. The natural HSA signal was more efficient than both α-factor and killer toxin, which was the least efficient of the series.

These observations show that it is difficult to predict how well a particular protein will be secreted by a homologous or heterologous signal peptide. The heterologous protein in this case, was most efficiently secreted using its natural signal peptide. However, the HSA signal was improved by inserting α-factor signal sequences, as was the killer toxin signal peptide. Other heterologous sequences have not been efficient in directing secretion of their native products and the introduction of a yeast signal has proved more successful. Different signals therefore, appear to work with varied efficiencies when fused to different proteins. The efficiency

of a particular signal peptide is likely to be related to its structural interaction with the heterologous protein itself and the overall interaction of the new combination with the secretory apparatus as a whole.

To summarize, in attempting to improve secretion levels of a heterologous protein in yeast, there are several parameters which can be altered and improved. Different signal peptides can be assessed to give the most efficient and correctly processed products. Further improvements in recovery levels can be sought by optimizing expression either by the choice of promoter or type of expression vector and translational efficiency can be optimized by choosing appropriate leader regions. Host strains can be manipulated to minimize proteolysis and supersecreting strains, which exhibit increased secretion efficiencies, have also been generated (Smith *et al*, 1985). These strains, including the *ssc*1 (or *pmr*1) mutation, appear to enhance secretion by overcoming in some way what is thought to be the rate limiting step in protein transport, ie. the ER to Golgi transfer step.

Other mutant strains can manipulate the glycosylation levels of heterologous proteins, eg. *mnn*9 mutants (section 1.6). Mammalian proteins, when produced in yeast, are often glycosylated to a far greater extent than in their normal hosts. This arises out of the fundamental differences in the glycosylation processes of higher and lower eukaryotes. Hyperglycosylation can cause problems for mammalian proteins, particularly those of potential therapeutic use in humans. This is due to problems that arise in the immunogenicity of the characteristic extended mannose chains of proteins produced in yeast (Melnick *et al*, 1990; Kurkuruzinski *et al*, 1987). Attempts to minimize this problem have included the alteration of the acceptor sequences by site directed mutagenesis to completely inhibit glycosylation and the production of the protein in glycosylation mutant strains such as *mnn*9 (Myajima *et al*, 1986; Ernst *et al*, 1987; Melnick *et al*, 1990). This approach has proved successful in the secretion of human α-antitrypsin in yeast (Moir and Dumais, 1987).

CHAPTER 2
MATERIALS AND METHODS

(2.1) **Materials**

Except where stated all chemicals were from Sigma Corporation, BDH and Reidel de Haen and were of AnalaR grade. Restriction enzymes, T_4 DNA ligase, Klenow polymerase, calf intestinal phosphatase and pUC19 DNA were from Boehringer Mannheim and Bethsheda Research Labs. (BRL). β-glucanase antiserum was a gift from Dr. E. Gormley, Trinity College, Dublin.

(2.2) **Table of strains.**

S.cerevisiae	Source
DBY746 Matα,*his*-Δ1,*leu*2-3,-112,*ura*3-52,*trp*1-289a.	D.C.U. stocks.
RSY11 Matα,*sec*18-1,*ura*3-52,*leu*2-3,-112,*suc*2.	R. Schekman, Univ. of California,
RSY12 Matα,*sec*53-6,*ura*3-52,*leu*2-3,-112.	Berkeley, U.S.A. "
RSY45 Matα,*sec*1-1,*ura*3-52,*leu*2-3,-112,*trp*1-289.	" "
RC631 Mata,*sst*2-1,*rme*,*ade*2-1,*ura*1,*his*6,*met*1	V.Bugeja, Maynooth College.
MD50 Matα,*pep*4-3,*leu*2-3,*leu*2-112.	J.R.Dickinson, Univ. of Wales, Cardiff.
E.coli	
JA221 F',*rec*A1,*leu*B6,*trp*Δ5,*hsd*MT,*hsd*R$^-$,*lac*Y,*xyl*.	D.C.U. stocks

(2.3) **Table of plasmids.**

(A). Yeast expression vectors:

pAAH5	Adh1 promoter, LEU2 marker *Hin*dIII cloning site.	Ammerer, G. (1983)
pAH9 pAH10 pAH21	pAAH5 derivatives, ATG start sites in 3 frames, no yeast terminator sequences.	Ammerer, G. (1983)
pVT102U	Adh1 promoter, URA3 marker.	Vernet *et al* (1987)

(B). Plasmids used in β-glucanase constructs:

pJG106	β-glucanase gene plus full signal in pUC8.	T. Ryan, D.C.U.
pJG104	β-glucanase gene minus signal in pUC8.	T. Ryan, D.C.U.
pJG104a	104 with *Hin*dIII linker in *Sma*I site.	This study
pJG104b	104 with *Hin*dIII linker in *Eco*RI site.	"
pJG104c	104b with *Hin*dIII linker in *Sma*I site.	"
p69A	MFα1 (α-factor) gene, signal and promoter in Yep13.	Kurjan and Herskowitz, (1982)
p69B	MFα1 promoter and signal in YEp13, *Hin*dIII cloning site.	"
p69D	p69B derivative, *Bam*HI linker inserted into *Hin*dIII.	T. Ryan, D.C.U.
pJG317	β-glucanase gene, full bacterial signal in pAAH5.	T. Ryan, D.C.U.

pJG205	β-glucanase gene, truncated bacterial signal in pAAH5.	T. Ryan, D.C.U.
pJG314	β-glucanase gene, α-factor promoter and promoter (in p69D).	"
pGS4	α-factor signal peptide on *Bam*HI/*Hin*dIII fragment in pBR322 derivative.	Shaw *et al*, (1988)
pUGS4	α-factor signal from pGS4 in pUC19.	This study
pJGS4	β-glucanase gene in pJGS4.	"
pVT314	β-glucanase gene, α-factor signal and Adh1 promoter.	"
pJBβ4	β-glucanase minus signal in pAH9 (pJBβ1, same construct with gene reversed).	"
pJBC55A	β-glucanase minus signal in pAAH5.	"
pRB155	Yeast β-actin gene in YEp24.	Shortle *et al*, (1982).

C. Plasmids used in B-glucuronidase constructs:

pRAJ260	β-GUS gene in pEMBL7.	Jefferson *et al*, (1986).
pRAJ260a	pRAJ260 with *Hin*dIII in *Eco*RI site.	This study
pJBU7	β-GUS gene in pAAH5.	"
pGUS1,2,3	β-GUS gene in 3 reading frames, in *Sma*I/*Eco*RI of pUC19.	T. Kavanagh T.C.D.

pGUS1a	pGUS1,2,3 with *Hin*dIII	This study
2a, 3a	linker in *Eco*RI site	
pGUS1b	pGUS1a with *Hin*dIII linker in *Eco*RI.	"
pGUS2c	pGUS2 with *Cla*I linker in *Sma*I site.	"
pGUSα6	β-GUS gene in p69B, pGUS1b fragment.	"
pΔ16.1	β-GUS deletion in pUC19.	"
pΔ16.9	β-GUS deletion in pUC19.	"
pJGUS6.1	β-glucanase/β-GUS fusion (plus bacterial signal).	"
pJGUS4.1	β-glucanase/β-GUS fusion (minus signal peptide).	"

(2.4) Media

All media was autoclaved at 15lbs/in^2 for 15 minutes.

LB (Luria Broth)

This media was used for routine culturing of *E.coli*.

Bacto-tryptone	10g
Yeast extract	5g
NaCl	10g
H_2O	to 1l
pH	7.5

For solid media Oxoid no.1 agar was added (13g/l).

YEPD

Complex media for routine culturing of yeast.

Yeast extract	10g
Bacto-peptone	20g
D-glucose	20g
H_2O	to 1l

For solid media Oxoid no.1 agar was added (20g/l).

MYGP

Complex media for culturing strains YT53 and YT54.

Malt extract	3g
Yeast extract	3g
Bacto-peptone	5g
D-glucose	20g
H_2O	to 1l

For solid media Oxoid no.1 agar was added (20g/l).

Yeast minimal medium

This synthetic medium was used for the selective growth of yeast auxotrophic strains carrying plasmids. Appropriate amino acid additions were made after autoclaving (see table below for details of stock solutions and working concentrations).

Difco yeast nitrogen base	6.7g
D-glucose[1]	20.0g
Succinic acid[2]	10.0g
NaOH[2]	6.0g
H_2O	to 1l

For solid media Oxoid no.1 agar was added (20g/l).

1.For liquid medium 50ml per litre 40% D-glucose was added after autoclaving.

2.Growth media for strains harbouring plasmids expressing β-glucanase were buffered to pH6.3 with succinic acid and NaOH.

Amino acids:

Stock solutions	Volume added per litre	Final conc. mg/ml.
2.0%(w/v) L-histidine	1.0ml	20.0
1.5%(w/v) L-leucine	2.0ml	30.0
3.0%(w/v) L-lysine	1.0ml	30.0
0.25%(w/v) Uracil	8.0ml	20.0
0.4%(w/v) L-tryptophan	5.0ml	20.0

Stock solutions were sterilised by autoclaving at 15lbs/in^2 for 15 mins. L-tryptophan was stored in the dark at 4°C.

(2.5) **Antibiotics**

For selective growth of transformed *E.coli* strains, appropriate antibiotics were added to media after autoclaving.

Ampicillin stock solutions (25mg/ml) were made in sterile H_2O and used at a working concentration of 40μg/ml for solid media and 35μg/ml for broths.

Tetracycline hydrochloride stock solutions (10mg/ml) were made up in 50% ethanol and stored in the dark at -20°C. The working

concentration used was 10ug/ml.
Chloramphenicol stock solutions (50mg/ml) were made up in 100% ethanol, stored at -20°C and used at a working concentration of 50 μg/ml for amplification of ColE1 plasmids.

(2.6) **Growth conditions and storage of strains.**
E.coli strains were grown at 37°C. Short term storage was on agar plates at 4°C. Long term storage was in 50% glycerol or 15% DMSO at -70°C.
Yeast strains were grown at 30°C (25°C for secretion mutants). Short term storage was on agar plates at 4°C. Long term storage was in 50% glycerol or 20% DMSO at -70°C.

(2.7) **Buffers and Solutions.**
Buffers for DNA manipulations:
TE buffer: 10mM Tris.HCl, pH8.0
1mM EDTA, pH8.0

Ligation buffer: 200mM Tris.HCl, pH7.6
100mM $MgCl_2$
10mM ATP
100mM DTT
DTT was stored as a 500mM stock at -20°C and added separately to ligation reactions.

Restriction buffers:
Buffers were supplied by the manufacturers and used according to their recommendations.

Nick translation buffer: 50mM Tris.HCl, pH7.2
10mM $MgSO_4$
1mM DTT
500ug/ml BSA

STE buffer: 10mM Tris.HCl, pH8.0
100mM NaCl
10mM EDTA

2X Bal31 buffer: 40mM Tris.HCl, pH7.2
25mM $MgCl_2$
25mM $CaCl_2$
2mM EDTA
1.2M NaCl

RNase (DNase free):
RNaseA (10mg/ml) was dissolved in 10mMTris.HCl (pH7.5), 15mM NaCl; heated to 100°C for 15 minutes ,allowed to cool slowly to room temperature and stored at -20°C.

Phenol/chloroform mix:
100g of phenol (AnalaR grade) was disolved in 100ml chloroform with 4.0ml isoamylalcohol and 0.8g 8-hydroxyquinoline. The phenol mix was stored under 100mM Tris.HCl, pH7.5, at 4°C in the dark.

Buffers for agarose gel electrophoresis:
10x TBE: 0.89M Tris. borate
0.89M Boric acid
0.02M EDTA
pH8.3
50x TAE: 2.0M Tris. borate
242g Glacial acetic acid
100ml 0.5M EDTA
pH8.0

5x Bromophenol blue: 0.25%(w/v) Bromophenol blue
25% Ficoll (type 400)

Solutions for isolation of DNA from agarose:

Sodium iodide solution:

90.8g NaI was disolved in 100ml H_2O. The solution was filtered through Whatman no.1 filter paper, 15g of Na_2SO_4 was added and the solution stored at 4°C in the dark.

Ethanol wash solution: 50% Ethanol
100mM NaCl
10mM Tris. HCl, pH7.5
1mM EDTA

This solution was stored at -20°C.

Solutions for preparation of RNA:

All solutions for preparation of RNA were made up in DEPC (diethyl polycarbonate) treated H_2O.

DEPC treatment:

DEPC (0.1%) was added to distilled H_2O, allowed to stand for 20 mins. at room temperature and autoclaved for 20 mins. at 15lb/in^2 to remove the DEPC.

Letts buffer: 0.1M LiCl
0.1M EDTA
0.01M Tris.HCl pH7.4
0.02% SDS

5x Formamide running buffer: 0.1M MOPS (pH7.0)
40mM Sodium acetate
5mM EDTA

Solutions for Northern blotting and hybridisation:

20x SSC: 175.3gNaCl
88.2g Tri-sodium acetate
H_2O to 1l
pH 7.0

Prehybridisation buffer: 6X SSC
2x Denhardts reagent
0.1% SDS
100μg/ml Salmon sperm DNA

50x Denhardts reagent: 5.0g Ficoll
5.0g Polyvinylpyrrolidone
5.0g BSA
H_2O to 1l

Buffers for dialysing yeast extracts:

5x PBS: 5.45g KH_2PO_4
10.7g Na_2HPO_4
45.0g NaCl
H_2O to 1l

Preparation of dialysis tubing:

Appropriate lengths of tubing were boiled for 15 mins. in 10mM EDTA, rinsed and boiled for 15 mins. in distilled H_2O.

Solutions for preparation of yeast extracts:

10x Lysis buffer: 50mM $NaPO_4$, pH7.0
10mM EDTA
0.1% Triton X-100
0.1% Sarkosyl
10mM β-mercaptoethanol

PMSF:

PMSF was made up freshly as a 10mM stock in 2-propanol and used as required.

DNS (3,5-dinitrosalicylic acid) solution for measuring β-glucanase activity:

5.0g DNS
1.0g Phenol
0.25g Na_2SO_3
100g Sodium potassium tartrate
2% NaOH
1l total volume

Buffers for polyacrylamide gel electrophoresis (PAGE):

SDS electrophoresis buffer:

25mM Tris. HCl, pH8.3
192mM Glycine
0.1% SDS

Solubilisation buffer:

100mM Dithiothreitiol (DTT)
80mM Tris. HCl, pH6.8
10% (w/v) Glycerol
2.0% (w/v) SDS
0.2% (w/v) Bromophenol blue

Coomassie blue stain:

0.5% Coomassie brilliant blue in acetic acid : water : methanol (1 : 10 : 8).

Destain solution: Acetic acid : water : methanol (1 : 10 : 8)

Solutions for Western blotting:

Transfer buffer: (per litre)

3.03g Tris. base
14.4g glycine
200ml methanol

TBST buffer:

150mM NaCl
10mM Tris. HCl, pH8.0
0.05% Tween-20

Blocking solution: TBST with 1% (w/v) BSA

AP (alkaline phosphatase) buffer: 100mM Tris. HCl, pH9.5
100mM NaCl
5mM $MgCl_2$

NBT stock: NBT (nitroblue tetrazolium), 50mg/ml in 70% N,N-dimethylformamide.

BCIP stock: BCIP (5-bromo-4-chloro-3-indolyl phosphate), 50mg/ml in 100% N,N-dimethylformamide.

AP colour development solution: 10ml AP buffer
66ul NBT stock
33ul BCIP stock

NBT was added to the AP buffer and mixed. BCIP was then added and mixed. The solution was made up freshly from stocks before use.

(2.8) Transformation of *E.coli*

Preparation of competent cells:

100ml of LB was innoculated with 1.0ml of an overnight culture of JA221 and grown at 37°C with aeration to an OD_{600}nm of 0.3 to 0.4. The culture was chilled on ice for 30 mins and cells were harvested by centrifugation at 10,000rpm for 10 mins at 4°C. The cells were washed in half the original volume of ice cold 50mM $MgCl_2$ and resuspended in half the original volume of 50mM $CaCl_2$. After incubation on ice for 30 mins, the cells were harvested as before and resuspended in one tenth the original culture volume of 50mM $CaCl_2$.

Transformation of competent cells:

Plasmid DNA (up to 250ng) was added to 200ul aliquots of competent cells in an eppendorf and incubated on ice for 60 mins. The cells were heat-shocked by placing them at 42°C for exactly 2 mins and returned to ice immediatly. 0.8ml of LB was added and the samples incubated at 37°C to allow expression of the plasmid encoded antibiotic resistant marker gene. The cells were plated in 200ul aliquots on LB agar containing the

appropriate selective antibiotic and incubated overnight at 37°C.

(2.9) **Transformation of** *S.cerevisiae*.

Preparation of yeast competent cells:

100ml of YEPD was innoculated with 50µl of an overnight culture of *S.cerevisiae* [1] and grown at 30°C with agitation to an OD600nm of 0.7. The cells were harvested at room temperature by centrifugation at 5,000 rpm. After 2 washes in half the original culture volume of TE, pH 7.5, the cells were resuspended in one tenth the original culture volume of 100mM LiAc and incubated at 30°C with agitation for 60 mins. The cells were harvested as before and resuspended in the same volume of 100mM LiAc. Routinely, 10ml samples of culture were used yielding 1.0ml competent cells.

1. When preparing and transforming competent cells of of *S.cerevisiae* secretion defective mutants, growth and incubation temperatures were 25°C.

Transformation of yeast competent cells:

Plasmid DNA (0.1µg-10µg) was added to 300µl competent cells. 0.7ml of 50% PEG_{4000} was added, mixed gently by inversion and incubated at 30°C for 60 mins. The cells were then placed at 42°C for 5 mins and plated directly onto selective media.

(2.10) **Generation of yeast spheroplasts.**

Cells were grown in YEPD to OD_{600} = 0.7, harvested and washed twice in 1.2M sorbitol. One tenth volume of zymolyase (3mg/ml) was added and the cells shaken (150 rpm) at 30°C for 60 mins. Spheroplast formation was monitored by adding a sample to H_2O and following the drop in OD_{600} readings as the cells lyse.

(2.11) **Small scale isolation of DNA from** *E.coli*.

Two methods for isolating small amounts of plasmid DNA were generally employed:

Method 1 (Birnboim and Doly 1979):

Cells were grown overnight at 37°C in LB with appropriate antibiotics. 1.5ml was pelleted by centrifugation and the

supernatant removed completely. The pellet was resuspended in 100 μl of Solution 1 and left on ice for 5 mins. 200μl of Solution 2 was added mixed, by inversion and incubated on ice for 10 mins. 150μl of Solution 3 was added, mixed by inversion and the sample left on ice for 5 mins. After centrifugation at 10,000 rpm for 5 mins, 400μl of the supernatant was removed to a fresh tube. An equal volume of phenol/chloroform was added, vortexed and spun . The aqueous layer was removed to a fresh tube and 0.8ml of ice cold ethanol was added. The sample was allowed to stand at room temperature for 10 mins. The DNA was pelleted by centrifugation at 10,000 rpm for 10 mins and the pellet washed in 70% ethanol. The DNA was dried under vacumn and resuspended in 30-50μl TE, pH 8.0. 5μl RNaseA (10mg/ml) was added and the DNA stored at 4°C.

Solution 1:

1.0ml 0.5M Glucose

1.0ml 0.1M EDTA

0.25ml 1M Tris.HCl,pH8.0

7.75ml H_2O

Solution 2: (made freshly every month and stored at RT)

2.0ml 1N NaOH

1.0ml 10% SDS

7.0ml H_2O

Solution 3: (3M Potassium acetate)

To 60ml of 5M potassium acetate, 11.5ml of glacial acetic acid and 25ml of H_2O were added.

Method 2 (Holmes and Quigley 1981) :

Cells were grown overnight at 37°C on LB agar containing the appropriate antibiotics. A patch of growth was transferred to an eppendorf using a sterile cocktail stick. The cells were resuspended by vortexing in 300μl STET buffer. 20μl lysozyme (10mg/ml) was added and the samples were allowed to stand at RT for 10 mins. The samples were placed in a boiling water bath for 60 seconds, centrifuged at 10,000 rpm for 5 mins. and the supernatant removed to a fresh tube. An equal volume of

isopropanol was added and the samples incubated at -20°C for 30 mins. The DNA pellet was washed with ether, dried and resuspended in 30-50μl TE buffer, pH 8.0. 5μl of RNaseA (10mg/ml) was added and the DNA stored at 4°C.

STET buffer: 8% w/v Sucrose
5% w/v Triton X-100
50mM EDTA
50mM Tris.HCl, pH 8.0

(2.12) **Large scale preparation of plasmid DNA from *E.coli*.**

250ml of LB (with the appropriate antibiotic addition) was innoculated with 1ml of an overnight culture of *E.coli*, and grown at 37°C with shaking to an OD600nm of 0.8. Chloramphenicol (1.0ml of 50μg/ml stock) was added to allow plasmid amplification and the cells were returned to 37°C for a further 12 to 16 hours.

Cells were harvested by centrifugation (10,000 rpm, 10 mins at 4°C) and resuspended in 2.0 ml sucrose solution. The cell suspension was transferred to a screw cap Beckman polycarbonate 50iTi tube and 0.4ml of lysozyme (20mg/ml in 0.25M Tris.HCl, pH 8.0) was added. After incubation on ice for 5 mins, 0.8ml of 0.25M EDTA was added, the cells mixed by inversion and incubated on ice for a further 10 mins. Triton-mix (3.2ml) was added, mixed and left on ice for 15 mins. When cell lysis was evident (indicated by an increase in viscosity of the mixture) the samples were centrifuged at 40,000 rpm for 20 mins at 4°C. The cleared lysate was removed to a 10ml tube and 6.7g of caesium chloride was added and mixed gently at room temperature. The solution was transferred to a 20ml Beckman quickseal polyallomer tube and 180μl of ethidium bromide (10mg/ml) was added. The total solution weight was brought to 14.1g using 10mM EDTA, pH 8.0. The tube was filled to the top with mineral oil heat sealed and centrifuged at 50,000 rpm for 24hrs at 18°C. The plasmid band was visualised by ultra violet transillumination and extracted from the tube using a sterile 18 guage needle and 1ml syringe.

Ethidium bromide was removed from the sample by extracting with isopropanol saturated with 20X SSC. The DNA was dialysed against TE pH 8.0 for at least 12 hrs with 3 changes of buffer. DNA was concentrated by ethanol precipitation.

Sucrose solution:		25% Sucrose
		50mM Tris.HCl, pH 8.0
Triton Mix:	5.0ml	20% Triton X-100
	12.5ml	0.25M EDTA
	2.5ml	1.0M Tris.HCl, pH 8.0

(2.13) **Separation of DNA molecules by agarose gel electrophoresis:**

Agarose gels were made up and run at constant voltage in 1X TAE or TBE buffer. 5X bromophenol blue dye was added to the samples before loading. Gels were stained in ethidium bromide (5μg/ml) for 20 mins, destained in H_2O for 10 mins and DNA was visualised by UV transillumination.

Gels were photographed using Kodak TriX-pan 35mm film. The film was developed with Kodak Universal developer (1/8 v/v in distilled H_2O) for 10 mins at 20°C and fixed in Kodafix (1/4 v/v in distilled H_2O) for 10 mins. Printing was on Kodak F_4 photographic paper using the above developer and fixer mixes.

(2.14) **General DNA manipulations.**

Restriction digestions:

Restriction buffers were supplied by the manufacturers and used under the recommended conditions. When restricted DNA was required for further manipulations it was cleaned up by extraction with phenol/chloroform and ethanol precipation or was passed through a sepharose column.

Ethanol precipitations:

For the routine precipitation of DNA 1/10th volume of 3M sodium acetate and 2 volumes of ethanol were added. After incubation at -20°C for at least 60 mins, the DNA was recovered by centrifugation at 10,000 rpm for 20 mins, the pellet washed once in 70% ethanol, dried under vacumn and resuspended in TE at the required pH.

Filling in 5' overhangs on DNA restricted DNA fragments:
Restricted DNA was incubated in 1X Klenow buffer, 2mM dNTPs and 1 unit of Klenow polymerase (1unit/μl) for 1 hour at 22°C. The reaction was stopped by heating to 70°C for 5 mins. Conditions were then adjusted for further manipulations.
Dephosphorylation of DNA:
1 unit of calf intestinal phosphatase (CIP) was added at the end of a restriction digest and the sample incubated at 37°C for 20 mins. The reaction was stopped by heating to 65°C for 5 mins after the addition of 1/10th volume 10X STE, 10mm EDTA and 1/20th volume 10% SDS (added to denature the enzyme). The DNA was then extracted with phenol/chloroform and ethanol precipated.
Addition of linkers:
Phosphorylated DNA linkers were ligated to blunt ended DNA in 1X LKB. Spermidine (final concentration of 10mM) and T_4 ligase (1Unit) were added and the reaction carried out at 22°C for 60 mins. 1Unit T_4 ligase was added and the reaction left at 12°C O/N.
Ligations:
Ligations were carried out at 12-14°C O/N in 1X LKB using 1U T_4 DNA ligase. Vector and insert DNAs were mixed in a ratio of approx. 1:10.

(2.15) **Bal31 digestions.**
Linearised DNA (30μg) disolved in TE pH 7.2, was mixed in an equal volume of 2x Bal31 buffer (total volume was usually 200μl). Bal31 enzyme (2.5 units) was added and the reaction incubated at 30°C. At appropriate time points, 20μl samples were removed into 2μl 0.25M EGTA on ice to stop the reaction. Because EGTA is a strong chelator of Ca^{2+} ions it does not inhibit restriction enzymes. Time points could therefore be analysed by restriction digestion directly after Bal31 deletion. For further manipulations,the DNA was cleaned up using sepharose CL6B columns.

(2.16) **Sepharose CL6B columns (pers.comm. T. Kavannagh,TCD).**

For cleaning up DNA preparations and changing buffer conditions samples were spun through mini-sepharose columns. The columns were prepared as follows : A hole was pierced in the base of a 0.75ml eppendorf. A small amount of glass beads (40 mesh) was placed in the tube to cover the hole and 0.5ml of 70% sepharose was added. The tube was placed in a 1.5ml eppendorf and spun at 1,500 rpm for exactly 2 mins. The liquid was removed from the large eppendorf and 20-50μl TE buffer added to the top of the column. The column was spun as before. This washing procedure was repeated once more. The column was then placed in a fresh, sterile 1.5ml eppendorf and the DNA added to the top of the column, the column spun and the DNA collected in the sterile 1.5ml eppendorf. In order to avoid increasing or decreasing the volume of the DNA the timing and speed of each spin was kept identical in each case.

(2.17) **Isolation of DNA fragments from agarose.**

The glass beads were prepared by T. Ryan (D.C.U.).

The DNA was separated by agarose gel (TAE) electrophoresis. The fragment of interest was cut out of the gel using a clean blade. The gel slice was chopped into small pieces, weighed and sodium iodide solution was added (2ml/g agarose). The sample was vortexed and incubated at 55°C for 5 mins or untill the agarose had disolved. 1-2μl of glass bead slurry was added, the sample vortexed and left on ice for 5 mins. The beads were spun at high speed for 10 secs and the supernatant removed. The glass bead pellet was washed 3 times in 50μl ice cold ethanol wash solution, spinning for 10 secs between each wash. The pellet was resuspended in 10-20μl TE, pH 8.0, and the DNA allowed to elute from the beads at 55°C for 3 mins. The beads were then spun and the eluate removed into a sterile eppendorf. This elution step was repeated to ensure maximum recovery of DNA.

(2.18) **Plate assay to detect β-glucanase activity.**

Yeast strains producing β-glucanase were grown on selective minimal media containing 0.05% lichenan. The medium was buffered using succinic acid and NaOH (section 2.7). Extracellular β-glucanase activity was detected by direct staining with congo red dye (0.1%). Intracellular activity was released by lysing the cells with a hot agar overlay containing 0.05% lichenan. The overlay was allowed to solidify and the plates incubated O/N at 37-45°C. β-glucanase activity was detected by staining with congo red dye (0.1%).

To assay cell supernatants and extracts, 6mm wells were cut in buffered agar plates containing 0.05% lichenan. The plates were incubated O/N at 37-45°C. To obtain clearer halos, plates were destained by rinsing in 1M NaCl.

(2.19) **DNS assay for measuring β-glucanase activity.**

The DNS assay (Miller *et al.* 1960) was used to accurately quantify β-glucanase activity. This assay measures the levels of reducing sugars produced by the enzyme from the β-glucan substrate. In order to ensure accurate quantitation of activity in cell supernatants, glucose present in the growth medium was removed before assaying, by dialysis. 2.0-5.0ml samples of supernatant were dialysed against PBS at 4°C for 12-16 hrs. The buffer was changed at least 3 times during that period. The substrate β-glucan was prepared by boiling β-glucan (1% w/v) in 0.1M sodium phosphate buffer, pH6.5 (section 2.7).

Assay: 1.0ml of substrate was prewarmed to 45°C for 10 mins before the addition of 1.0ml enzyme sample. Each sample was mixed by vortexing and incubated at 45°C for 20 mins exactly. The reaction was stopped by cooling under running water for 15 secs. and the addition of 3.0ml DNS solution. Blanks were prepared by mixing 1.0ml substrate, 1.0ml enzyme sample and 3.0ml DNS solution. All samples were boiled for 15 mins exactly and cooled under running water. 5.0ml H_2O was added and the absorbance at 540nm measured against the stopped blanks. The amount of reducing sugars present was read from a standard curve prepared using glucose standards.

(2.20) **Preparation of yeast cell extracts for DNS assays.**

5 X 10^7 cells were harvested by centrifugation (5,000 rpm, 5 mins) and resuspended in 1X lysis buffer (section 2.7), 10 mM PMSF. Lysis was typically carried out in a volume of 5.0ml. 1.0g glass beads was added and the cells were vortexed for 3 mins (6 X 30 second bursts with 30 seconds on ice between each burst). The cell debris was spun out and the cleared lysate removed to a fresh tube. For smaller preparations, volumes were adjusted appropriately. (In this procedure, 40 mesh glass beads were used for lysing the cells). * see end of methods section (pg 78).

(2.21) **Separation of proteins by polyacrylamide gel electrophoresis (PAGE).**

Preparation of running gel:

	8%	10%	15%
Acrylamide:bis-acrylamide (29:1)	5.30ml	7.00ml	10.30ml
1.87M Tris.HCl,pH 8.8	4.20ml	4.20ml	4.20ml
10% SDS	0.12ml	0.12ml	0.12ml
10% Ammonium persulphate	0.10ml	0.10ml	0.10ml
TEMED	0.02ml	0.02ml	0.02ml
H_2O	11.10ml	9.40ml	6.10ml

Preparation of stacking gel (3%):

Acrylamide:bis-acrylamide	2.50ml
0.5M Tris.HCl, pH 6.8	1.20ml
10% SDS	0.10ml
10% Ammonium persulphate	0.10ml
TEMED	0.10ml
H_2O	7.50ml

(2.22) **Preparation of yeast cell extracts and supernatants for PAGE.**

Yeast cells were lysed as per section 2.20. 1.0ml samples of extract and supernatant were dialysed O/N against 1mM $NaPO_4$, pH 7.0, freeze dried and resuspended in 1X solubilisation buffer (20-100μl). Samples were boiled for 3 mins before loading.

(2.23) **Western blotting procedure.**

Transfer of proteins to nitrocellulose:

Following electrophoresis, proteins were transferred to nitrocellulose using a Bio-Rad transfer cell. Gels were equilibrated in transfer buffer (section 2.7) for 20 mins before transfer. The transfer unit was assembled as follows: the fibre pads, Whatmann paper and nitrocellulose were soaked in transfer buffer . A fibre pad was placed on the cathode panel of the gel unit. A piece of Whatmann no. 2 paper, cut to the size of the gel was placed on top. This was followed by the gel, a sheet of nitrocellulose, another piece of Whatmann paper and the second fibre pad. The unit was closed and placed in the transfer tank. Transfer was carried out in transfer buffer at 30V,0.1A O/N or at 60V, 0.21A for 4 hrs. A small stirring bar was used to circulate the buffer throughout transfer in order to improve heat transfer and ensure a homogenous pH throughout the tank. Efficiency of transfer was monitored by the use of pre-stained markers (Sigma). After transfer was complete, the membrane was used directly for binding to antibodies or was sealed in plastic and stored at 4°C. At no stage was the membrane allowed to dry out.

Binding of primary antibody and secondary enzyme-conjugated antibody to blotted proteins:

The membrane was submerged and rinsed in TBST. The TBST was replaced with blocking solution and incubated at room temperature with gentle agitation for 30-60 mins. The blocking solution was decanted and replaced with TBST ($0.1ml/cm^2$) containing the primary antibody ($20\mu l$ for β-glucanase). After inbcubation at room temperature for 60 mins, the membrane was washed 3 times in TBST (5-10 mins per wash). The membrane was transferred to TBST containing a 1:7,500 dilution of anti-rabbit IgG alkaline phosphatase (AP) conjugate for β-glucanase, or anti-rat IgG AP conjugate for β-glucuronidase. This was incubated for 60 mins at room temperature and washed 3 times in TBST (5-10 mins per wash).

Development of AP colour reaction:

The membrane was blotted dry on filter paper and transferred to

10ml of AP colour development solution (prepared as described section 2.7). Colour development was continued until bands of the desired intensity appeared. The reaction was stopped by rinsing for several minutes in distilled H_2O. The membrane was air dried and stored, sealed in plastic.

(2.24) **β-glucanase activity gel.**

Yeast cell extracts and supernatants were prepared and subjected to SDS PAGE as described in sections 2.21 and 2.22. After electrophoresis, the gel was renatured in 50mM $NaPO_4$, 1% SDS for 60 mins at room temperature. The gels were agitated gently and the renaturation buffer changed at least 3 times during incubation. The gel was then placed on top of a second acrylamide gel containing 0.15% lichenan (buffered with Succinic acid and NaOH), and left at 25°C for 24hrs. The lichenan gel was stained in congo red dye. Destaining was in 1M NaCl. Bands of β-glucanase activity appeared as white on a red background. To obtain a clearer background, the lichenan gel was made from a stock solution of lichenan (0.3%), that had been boiled in H_2O for 10mins. The insoluble lichenan particles were spun out of the solution before adding to the polyacrylamide gel.

(2.25) **Isolation of RNA from *S.cerevisiae*.**

Total RNA was isolated from yeast using a method adapted from Sherman *et al.* (1986).

In order to minimize RNase activity during the isolation of RNA the following precautions were taken. All solutions (section 2.7) used in this procedure were made up in DEPC treated H_2O and autoclaved at 15lb/in^2 for 15 mins. Glassware and glass beads were washed in 3M HCl and baked at 180°C for 8 hrs before use. Plasticware was rinsed in chloroform and autoclaved before use.

Cells were grown in appropriate media to an OD600nm of approximately 1.0. 100ml of cells were harvested by centrifugation at 5,000 rpm for 10mins at 4°C and resuspended in 2.0ml Letts buffer. The cell suspension was transferred to a

15ml corex tube containing 8g glass beads (40 mesh) and 2ml phenol/chloroform that had been equilibrated with Letts buffer. The cells were lysed by vortexing for 3 mins (6 x 30 sec. bursts with 30 secs intervals on ice between each burst). 2.0ml Letts buffer was added, the sample mixed by vortexing and spun for 5 mins at 8,000 rpm at 4°C. The aqueous layer was removed and the sample extracted 3 times with phenol/chloroform The RNA was precipitated in 0.1% 5M LiCl and 2 volumes ice cold absolute ethanol and incubated at -20°C for at least 3 hrs. Total RNA was harvested by centrifugation at 10,000 rpm for 10 mins at 4°C. The pellet was washed in 80% ethanol, air dried and resuspended in 1.0-1.5ml DEPC treated H_2O. RNA concentrations were estimated at OD260nm.

(2.26) **Formaldehyde gel electrophoresis.**

RNA molecules were separated on formaldehyde gels before blotting onto nitrocellulose.

(a) Preparation of formaldehyde gel: For a 200ml gel (1.2%), 2.4g agarose was boiled in 124ml DEPC treated H_2O and allowed to cool to 60°C. 40ml 5X formaldehyde buffer (section 2.7) and 36ml 37% formaldehyde was added and the gel allowed to set at room temperature.

(b) Preparation of RNA samples for loading: For a total sample volume of 20μl, 4μl 5X formaldehyde buffer, 5μl formamide, 2μl 37% formaldehyde and 1μl ethidium bromide (1mg/ml) were mixed in an eppendorf. Up to 8μg of RNA was added. A separate sample containing 4μl 5X bromophenol blue and 4μl H_2O and no RNA was also prepared as a marker for running the gel. The samples were heated to 65°C for 10 mins before loading.

The gel was run at 3V/cm^2 in 1X formaldehyde buffer for 4-6hrs until the dye had reached half way down the gel.

(2.27) **Transfer of RNA to nitrocellulose.**

After staining, the gel was soaked in 0.05N NaOH to partially hydrolyse the RNA, rinsed in DEPC treated H_2O and soaked in 20X SSC for 45 mins.

Bi-directional transfer of RNA to nitrocellulose: An 8cm high

stack of paper towels was placed on a glass plate. Two pieces of Whatmann no.2 paper, soaked in 2X SSC and cut to the dimensions of the gel, were placed on top. A sheet of nitrocellulose, soaked in 20X SSC was placed on top of the filter paper. This was followed by the gel, a second presoaked sheet of nitrocellulose, 2 pieces of Whatmann paper (soaked in 2X SSC), an 8cm stack of paper towels and finally a glass plate. To ensure even transfer the stack was inverted 4 times in the first 30mins and transfer allowed to continue for 12hrs. After transfer, the towels and Whatmann paper were removed and the positions of the wells marked on the nitrocellulose. The nitrocellulose filters were washed in 6X SSC at room temperature and allowed to air dry for approximately 60 mins. The gel was stained again in ethidium bromide to assess efficiency of transfer. The filters were placed between 2 sheets of Whatmann No.1 paper, baked for 2hrs. at 80°C and stored at room temperature sealed in plastic.

(2.28) **Random primer labelling of DNA.**

Random primer labelling of probe DNA was carried out using a Promega "Prime a gene" kit.

25ng of DNA was denatured by heating to 100°C for 2 mins and transferred to ice. The DNA was mixed with 10μl 5X Labelling buffer, 2μl 500μM dNTPs and 2μl 1mg/ml acetylated BSA. 5μl [α^{32}P] dATP and 5μl Klenow polymerase were added and the volume made up to 50μl with H_2O. The reaction was mixed gently and incubated at room temperature for 60 mins. The reaction was terminated by heating to 100°C for 2 mins and chilled on ice. 2μl 0.5M EDTA was added and the labelled, denatured probe DNA was used directly for hybridisation to RNA filters.

(2.29) **Hybridisation of probe to RNA blots.**

The baked filter was floated on the surface of 6X SSC until wet, submerged and allowed to soak for 2 mins. The filter was then placed in a plastic bag and pre-hybridisation mix (section 2.7) added to volume of 0.2ml/cm^2 nitrocellulose. The bag was resealed, eliminating as much air as possible and placed at

68°C for 2 hrs with shaking. After pre-hybridisation, the probe was added to the filters, the bag resealed and hybridisation allowed to take place at 68°C for 16-24 hrs. After hybridisation, the filters were washed for 20 mins at room temperature for 20 mins in 1X SSC, 0.1% SDS. This was followed by 3 washes of 20 mins each in 0.2X SSC, 0.1% SDS at 68°C. After washing, the filter was air dried for 60 mins, sealed in plastic and exposed to X-ray film (Kodak OM84) with intensifying screens at -70°C for up to 1 week. The films were developed in Kodak LX24 developer for 5 mins at room temperature. Fixing was in Kodak FX40 fixer, 2 X 2 mins at room temperature. The films were rinsed in water and allowed to dry.

(2.30) **β-glucuronidase assays.**

Spectrophotometric assay:

Samples of yeast cell extracts and supernatants were incubated in 1X reaction buffer (section 2.28) and 1mM p-nitrophenyl glucuronide (PNPG) in a total volume of 1.0ml at 37°C. The reaction was stopped by the addition of 0.4ml 2.5M 2-amino,2-methyl propanediol at intervals of 30, 60 and 90 mins for any one assay (samples were prepared in triplicate). The absorbance at 450nm was measured against a substrate blank for each time point and a standard curve for each sample was constructed (OD450nm vs. time). The amount of β-glucuronidase in a sample was calculated. One unit of β-glucuronidase enzyme is defined as the amount that will produce 1.0 nmole of p-nitrophenol per minute at 37°C. The molar extinction coefficient of p-nitrophenol is 14,000. In a total volume of 1.4ml, an absorbance of 0.01 is equivalent to 1.0 nmole of product. 1.0 nmole of product is equivalent to 5.0ng of β-glucuronidase enzyme.

Flourimetric assay:

E.coli strains expressing β-glucuronidase from multicopy plasmids were identified by spraying colonies growing on agar plates with 1mM MUG (4-methyl umbelliferyl glucuronide). Plates were then incubated for 30 mins. at 37°C and viewed by UV transillumination.

(2.31) **β-glucuronidase activity gel.**

SDS polyacrylamide gels were renatured in 1X reaction buffer (50mM $NaPO_4$ pH 7.0, 0.1% Triton X-100, 10mM EDTA, 10mM β-mercaptoethanol) for 60 mins at room temperature with gentle agitation. The buffer was changed at least 3 times during this period. The renatured gel was transferred to 1X reaction buffer containing 0.5mM MUG (4-methyl umbelliferyl β-D-glucuronide) and incubated at 37°C for 30 mins. The gel was washed in 0.2M $NaCO_3$ to stop the reaction and to enhance fluorescence. β-glucuronidase activity was viewed by UV transillumination and appeared as fluorescent bands.

(2.32) **Incorporation of Mata cells into MYGP agar.**

RC631 cells were grown to an OD_{600} = 1.0 units. 1.0ml of a 10^{-2} dilution of the cells was added to 10ml of molten MYPG agar (at 45°C), mixed and poured into a petri dish. The agar was allowed to set at room temperature before use.

* Cell lysis was monitored microscopically and was standardised using the Bradford assay for measuring total cell protein. Based on the total protein / dried weight ratios obtained, efficient cell lysis was achieved routinely.

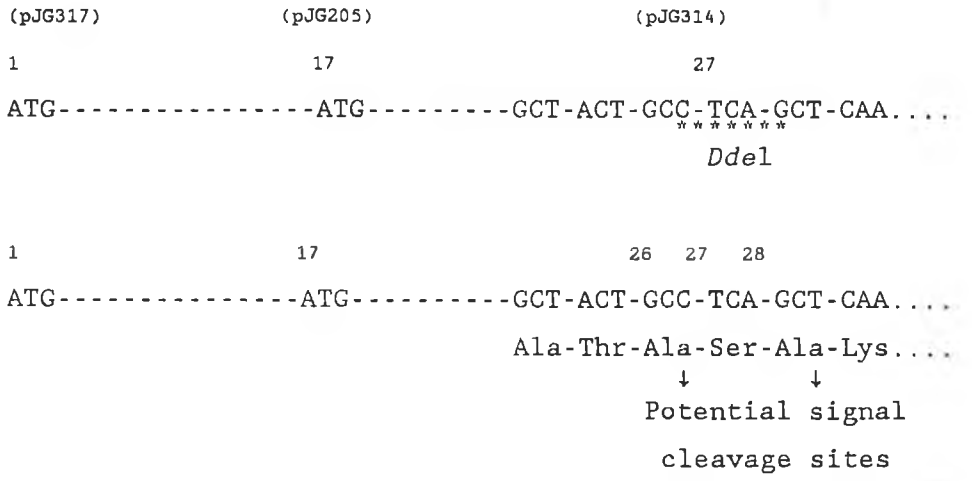
CHAPTER 3
RESULTS

Section 3.1
Analysis of β-glucanase production and secretion in *S.cerevisiae*.

(3.1) Introduction.

Endo (1-3), (1-4) -β -D -glucanase, from *B.subtilis* (from now on referred to as simply β-glucanase), hydrolyses β-1,4 glucosidic bonds provided they are adjacent to a β-1,3 glucosidic bond. These type of bonds comprise the major type of linkages in the glucans of malt and barley cell wall endosperm. In the brewing process, these glucans are normally degraded by endogenous barley and malt β-glucanase enzymes. However, the high temperatures associated with mashing and kilning procedures destroys these enzymes and this can result in the glucans being extracted in the wort. The presence of high molecular weight glucans in the wort can ultimately cause a reduction in beer quality. *The B.subtilis* β-glucanase enzyme cleaves the same β-1,4 linkages and produces a similar range of products from malt and barley β-glucans as the cereal enzymes. The *B.subtilis* gene was cloned with the intention of producing a yeast strain that could hydrolyse these excess β-glucans during the fermentation process (Cantwell and McConnell 1983). The cloned gene has a coding sequence of 726bp (app.1), producing a protein of 242 amino acids (Murphy *et al*. 1984). This sequence includes a signal peptide of 26 -28 amino acids. The signal sequence is characteristic of most signal peptides in that it includes a short hydrophillic domain of six amino acids, followed by a hydrophobic sequence. The signal ends with two potential cleavage sites as shown below (Fig.3.1). A useful feature of the cleavage site is the presence of a *Dde*I site which allows the convenient separation of signal and mature sequences during manipulations.

Figure (3.1).Features of the β-glucanase signal peptide.



The entire β-glucanase gene, including its signal peptide and terminator sequence, were inserted into the *Bam*HI site of pUC8 (T.Ryan, D.C.U.). This plasmid, pJG106, has formed the basis for many of the manipulations to follow and is shown in app.(3). The β-glucanase gene in its entirety can be removed on a *Bam*HI or *Hin*dIII fragment.

The β-glucanase gene was expressed in *S.cerevisiae* under control of the α-factor promoter and signal peptide sequence in pJG314. (Fig.3.2) (T. Ryan, D.C.U.). To generate this construct, the β-glucanase gene was removed from its signal peptide sequences at the *Dde*I site, BamHI linkers were added and the gene inserted into the *Bam*HI site of p69D (a derivative of p69A, see app.(2) for details) (Kurjan and Herskowitz, 1982), generating an in-frame fusion with the α-factor signal peptide. When transformed into DBY746, pJG314 was shown to secrete β-glucanase into the yeast cell medium.

In addition to pJG314, two further constructs pJG317 and pJG205, were made. pJG317 contains the entire β-glucanase gene, including the bacterial signal peptide, under ADH1 promoter control in pAAH5 (Fig. 3.3). Using this construct, it was shown that the heterologous *Bacillus* signal sequence also secretes the β-glucanase enzyme in *S.cerevisiae* (T. Ryan, D.C.U.).

The β-glucanase signal peptide has a second ATG start site (at

amino acid position 17), in frame with the initiation codon. The gene including the signal peptide from this second ATG, was introduced into *S.cerevisiae* under control of the ADH1 promoter in pAAH5 (Cantwell *et al.* 1986). This plasmid, pJG205 (Fig.3.3), was also shown to produce and secrete the enzyme despite its truncated signal sequence. Initial investigations suggested, that in terms of secretion of the enzyme in *S.cerevisiae*, pJG317 and pJG205 were more efficient than pJG314 (T.Ryan, pers. comm.). In fermentation trials, an ale and lager brewing strain transformed with pJG205(1) (pJG205 modified by the insertion of the CUP1 dominant selectable marker), also secreted the enzyme in sufficient quantities to significantly reduce the levels of β-glucan during the fermentation process (Cantwell et al. 1985).

The work presented here was undertaken to examine in more detail the production and secretion of the *B.subtilis* β-glucanase enzyme in *S.cerevisiae* using these three plasmids. Of particular interest was to determine whether the extracellular levels of activity observed in pJG317 and pJG205, were due to translocation into and transport through the conventional yeast secretory pathway, rather than simply due to cell lysis or leakage. Also, it was hoped to examine the efficiency of the bacterial signal and assess its potential for use as a general secretion sequence for heterologous protein production in yeast. Examination of the *Bacillus* protein product, with respect to processing and modification, was also carried out. The difficulties associated with rapid quantitation of β-glucanase levels prompted the investigation of another heterologous protein, β-glucuronidase (an *E.coli* enzyme), and its potential for development as a reporter enzyme for *S.cerevisiae*.

Summary of plasmid constructs:

CONSTRUCT	PROMOTER	SIGNAL PEPTIDE
pJG205	ADH1	Truncated β-glucanase
pJG317	ADH1	Complete β-glucanase
pJG314	α-factor	α-factor

NOTE: Except where indicated, pJG317, pJG205 and pJG314, refers to these plasmids transformed into DB746.

Figure (3.2) pJG314

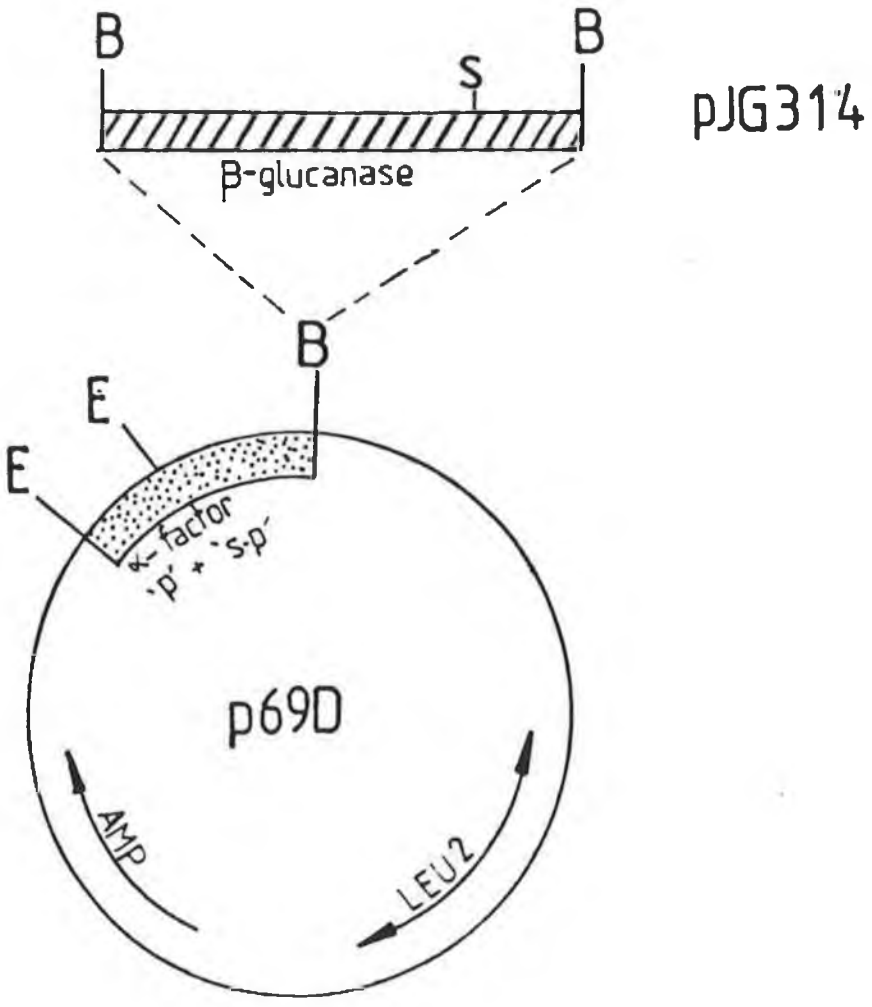
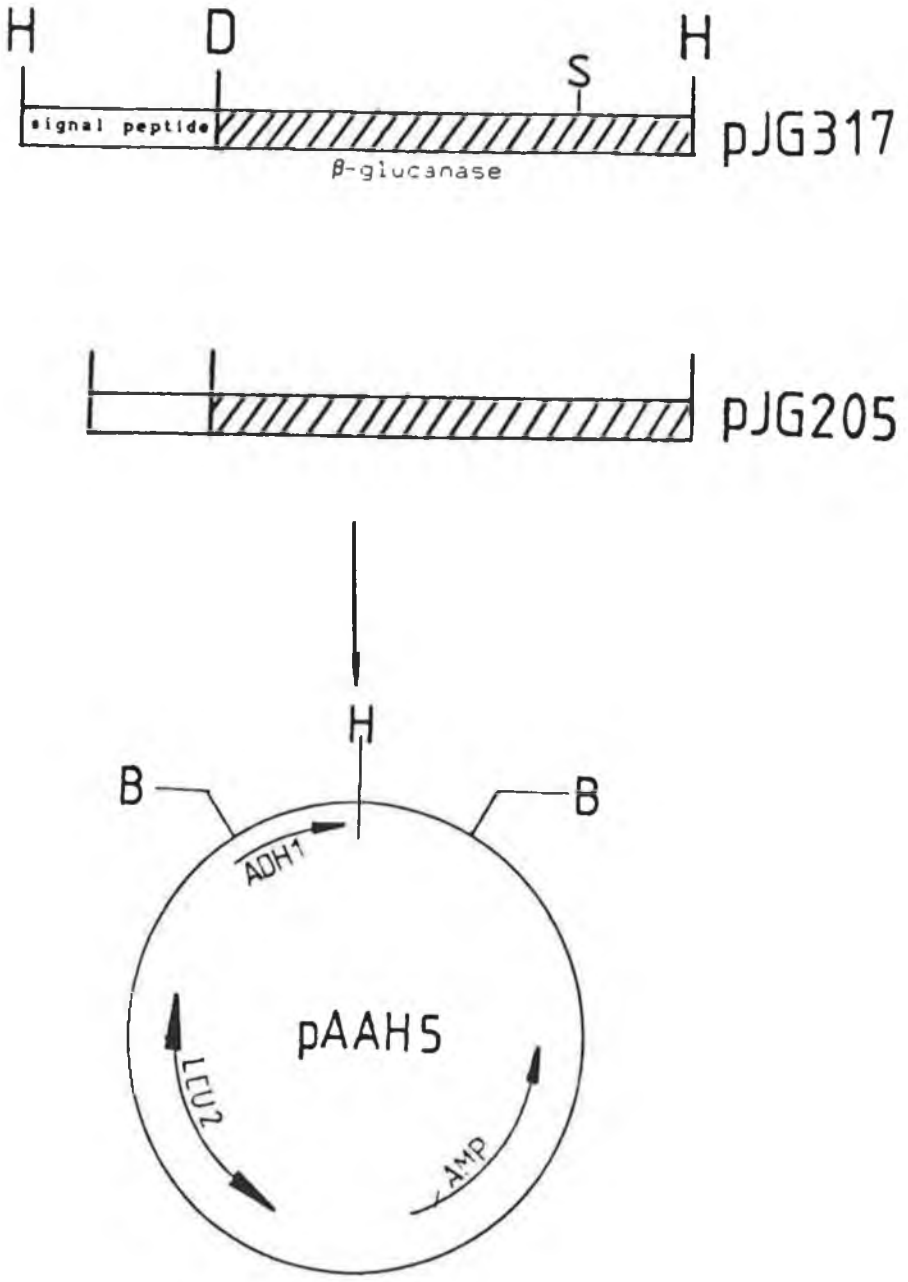


Figure (3.3). pJG317 and pJG205.



(3.2) **The β-glucanase plate assay.**

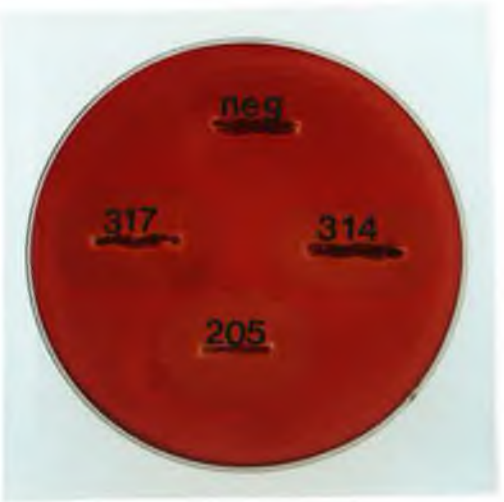
The plate assay for detecting β-glucanase activity (section 2.18), which was developed to screen for *E.coli* cells producing the enzyme (Cantwell and McConnell 1983), can also be used to identify and monitor β-glucanase production in yeast cells. The substrate lichenan (from *Usnea barbata*), included in plates at a concentration of 0.05%, is composed of glucans with both β-1,3 and 1,4 linkages. These linkages are specifically broken down by the *Bacillus* β-glucanase, producing a halo visible on staining with congo red dye (0.1%). Non-selective media such as YEPD and MYGP, containing lichenan, stain with congo red. Minimal medium however, must be buffered in order to detect extracellular β-glucanase production under selective growth conditions (section 2.4).

Intracellular and extracellular levels of β-glucanase activity were compared in pJG317, pJG205 and pJG314 using the plate assay (Figure 3.4). This qualitative assessment suggests that all three constructs produce similar overall levels of the enzyme, with a slightly larger halo in pJG205 at an intracellular level. The extent of this increase in activity is demonstrated quantitatively in section (3.4) and reflects the limitations of the plate assay at high concentrations of β-glucanase.

Figure (3.4).

(a) Extracellular levels of β-glucanase activity detected by direct staining with congo red.

(b) Intracellular levels of activity were released using a hot agar overlay and stained with congo red.



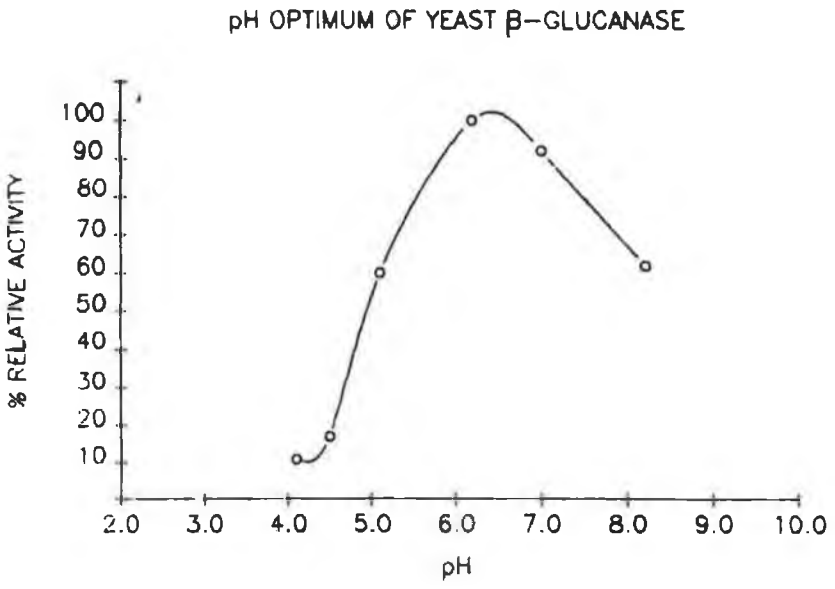
(3.3) The DNS assay for measuring β-glucanase activity.

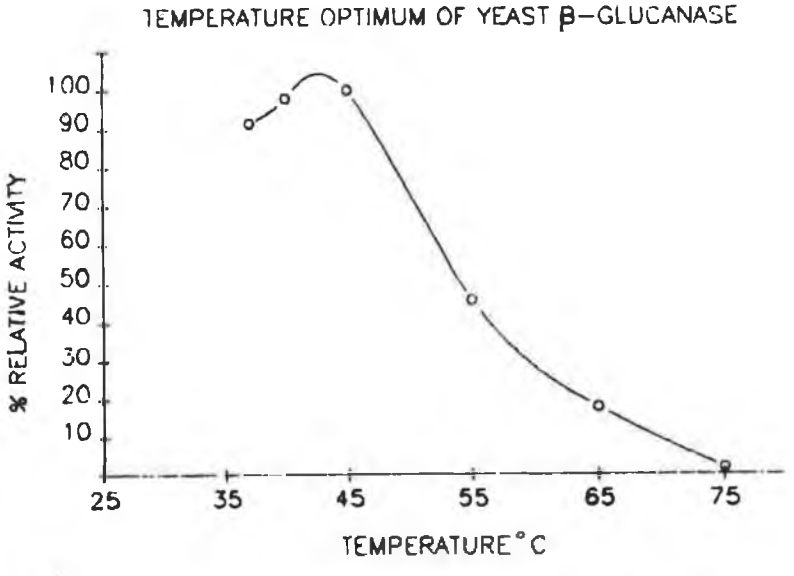
β-glucanase hydrolyses the substrate β-glucan, to produce dextrins with reducing ends. The production of these reducing sugars can be measured using the DNS assay developed by Miller (1960). The method employed for accurate quantitation of β-glucanase activity using the DNS assay is described in section (2.19).

The optimum assay conditions for the commercial *Bacillus* β-glucanase enzyme, recommended by the manufacturers (Novo Cereflo), is at a pH of 5.5 and an incubation temperature of 55°C. The pH and temperature profiles of β-glucanase produced in *S.cerevisiae* (DBY746) were examined. Extracts were prepared from pJG317 (OD_{600}=1.0) and β-glucanase activity measured over a range of pH and temperature values. Assays were carried out in duplicate and the pH and temperature profiles below represent an average of two values (Figure 3.5). 45°C at pH6.5 was shown to be optimum for the β-glucanase enzyme when produced in yeast and subsequent liquid assays were carried out under these conditions.

Figure (3.5).

pH and temperature profiles of *β*-glucanase produced in *S.cerevisiae*.





(3.4) β-glucanase production in pJG317, pJG205 and pJG314: comparing intracellular and extracellular levels of activity.

In order to accurately assess and compare the levels of β-glucanase produced in pJG317, pJG205 and pJG314, intracellular and extracellular enzyme activity was monitored over the complete growth cycles of the three strains using the DNS assay. 1.0ml of a stationary phase culture was innoculated into 250mls of minimal medium and shaken (150 rpm) at 30^{0}C. At 3 hourly intervals, 5.0ml of culture was removed and chilled on ice. An OD_{600} reading was recorded at each time point and extracts and supernatants were prepared and assayed for β-glucanase activity as described in sections 2.19 and 2.20. The results of the growth curves are shown (Figure 3.6(a), (b) and (c)).

Figure (3.6)a

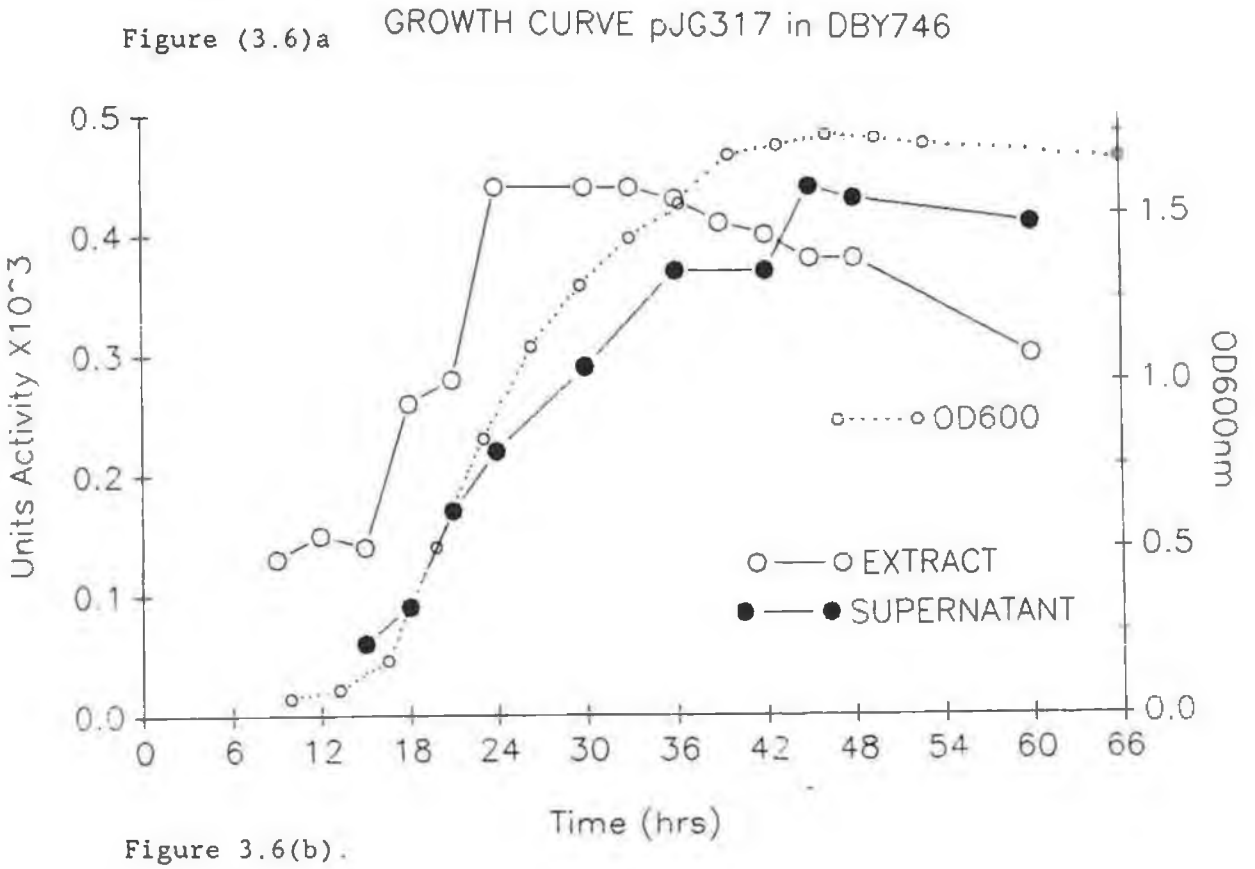


Figure 3.6(b).

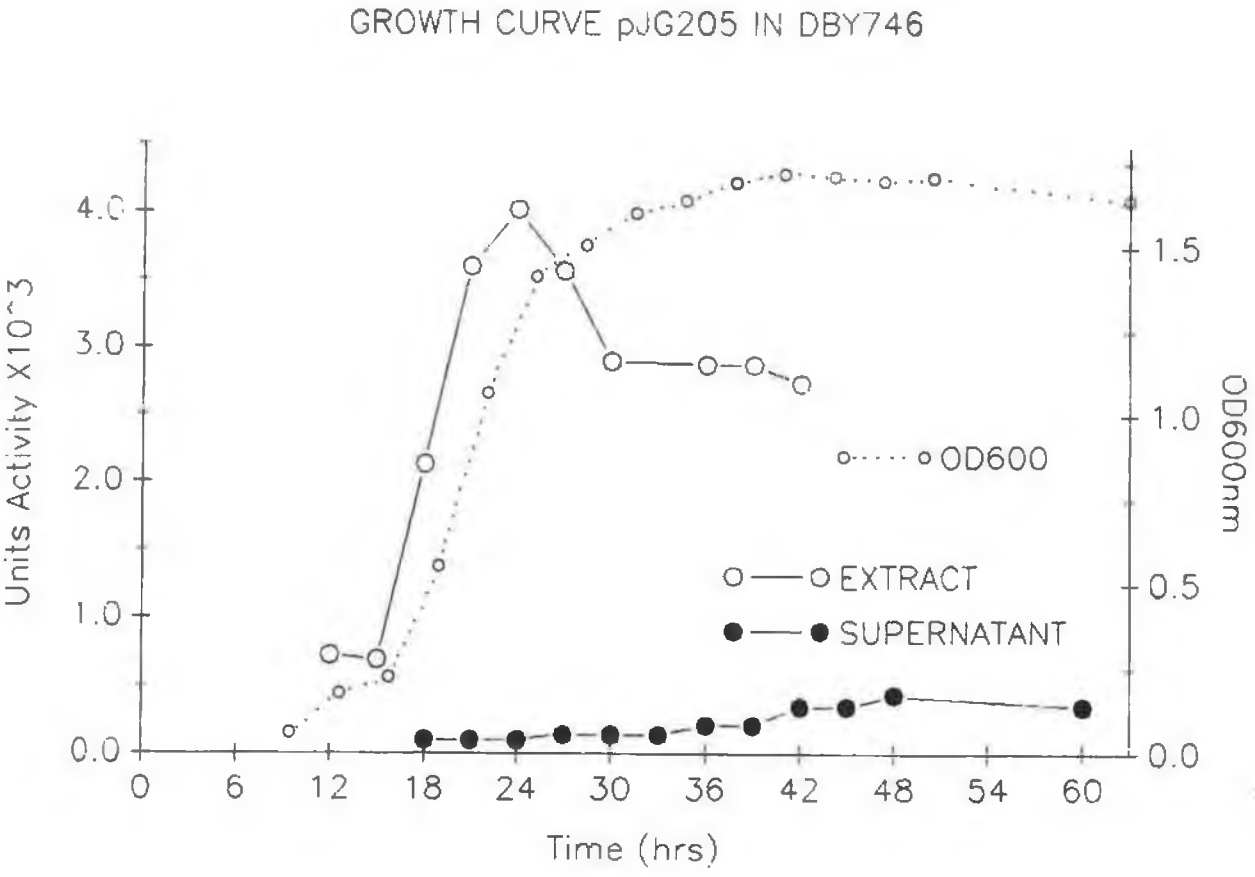


Figure 3.6(c). GROWTH CURVE pJG314 in DBY746

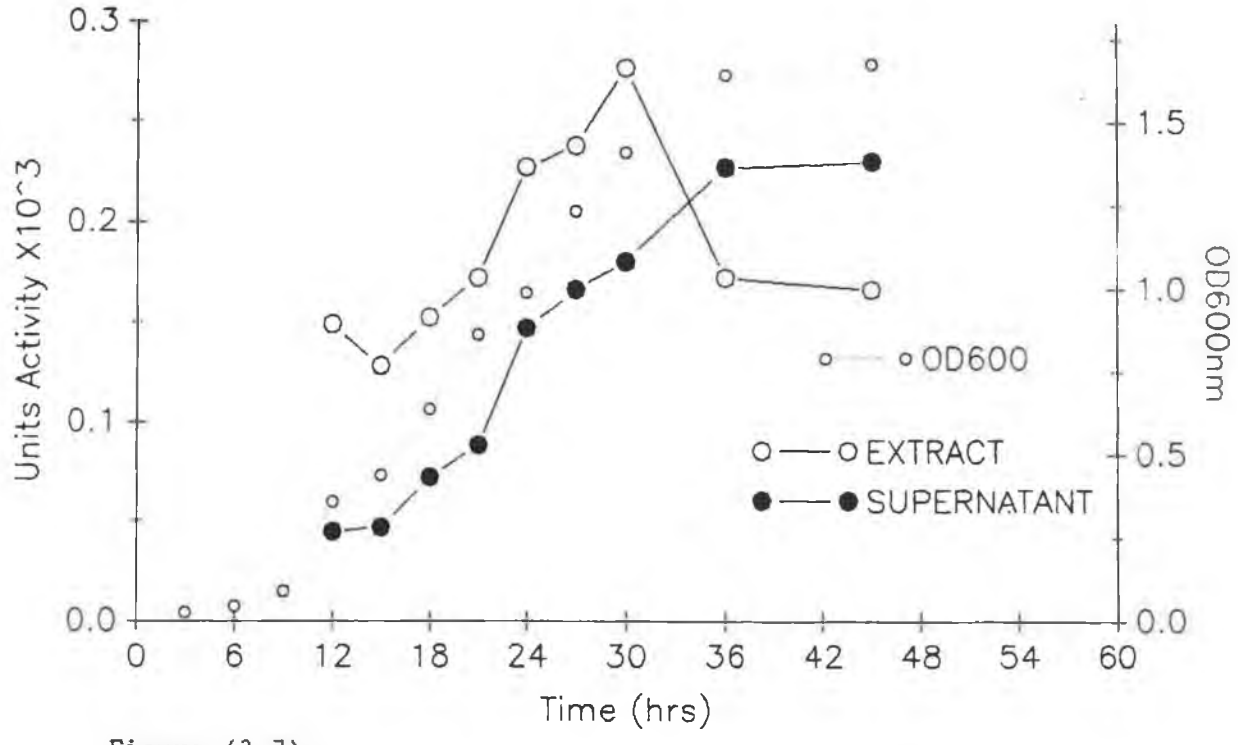
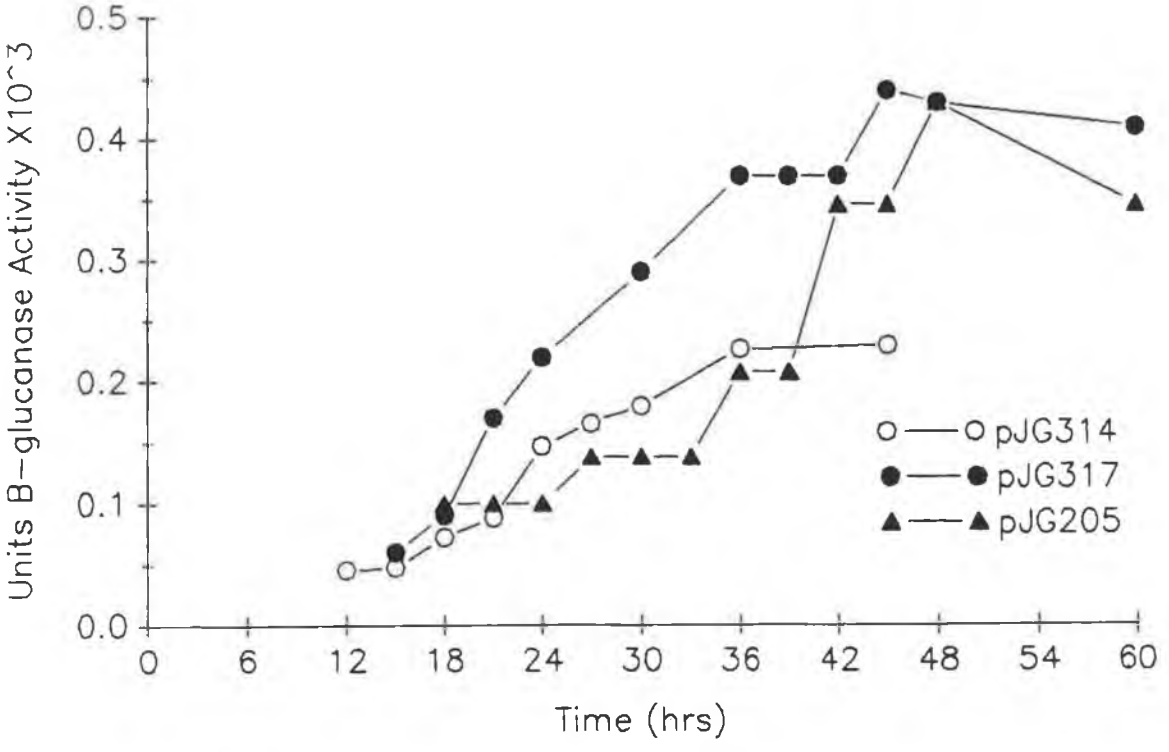


Figure (3.7).

SECRETED B-GLUCANASE IN pJG314, pJG317 and pJG205



The growth curves show that enzyme production and secretion closely follows cell growth. Secretion of the enzyme continues on into stationary phase for up to 10 hours after production has ceased. The most striking aspect of comparing the three constructs is the large differences in intracellular levels of β-glucanase activity observed in pJG205 compared to pJG317 and pJG314. pJG205, containing the truncated bacterial signal peptide, produces almost nine times as much enzyme activity at an intracellular level as pJG317. Comparison of secreted levels (Figure 3.7, above) shows, that while pJG317 secretes higher levels of the enzyme than pJG205, this difference in extracellular levels cannot account for the extent of the accumulation observed in pJG205. The increase in intracellular levels of activity in pJG205, could be due to transcriptional, translational or post-translational events, and these possibilities are discussed at a later stage.

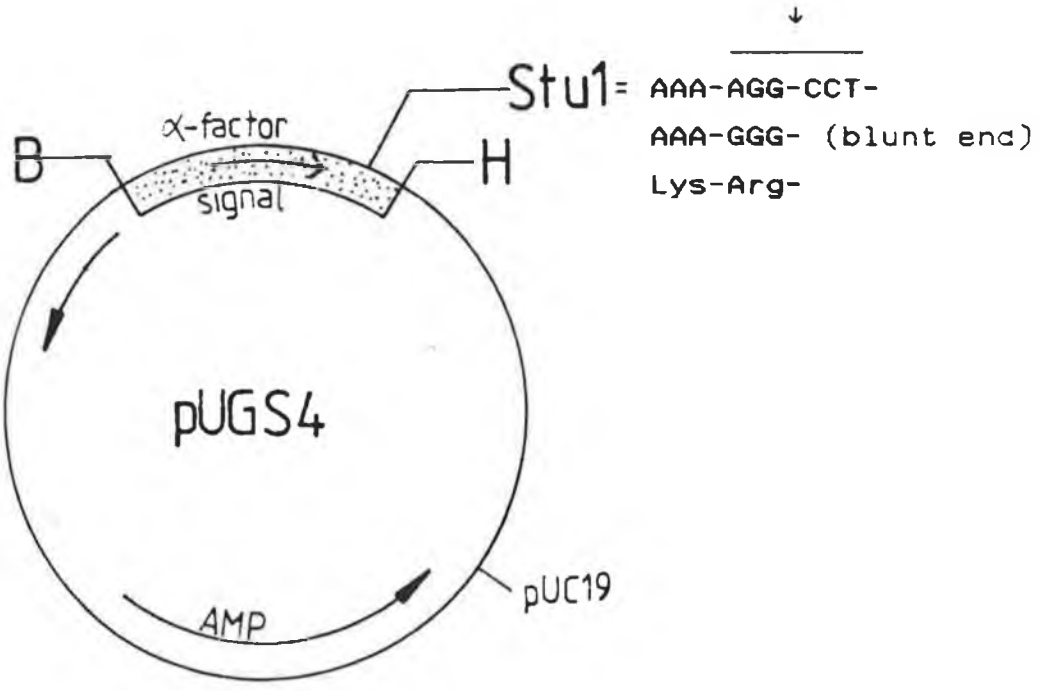
Secretion levels in pJG314 are lower than both pJG317 and pJG205, possibly reflecting differences in processing of the two signal peptides, (α-factor by the KEX2 cleavage enzyme and the bacterial signal by signal peptidase). Alternatively, taking into account the lower intracellular levels also observed in pJG314 (i.e. 0.3×10^3 U/ml in 314 compared to 0.5×10^3 in 317), this may be a reflection of the relative strengths of the ADH1 and α-factor promoters.

(3.5) Construction of pVT314 : comparison of the bacterial and yeast signal peptides under the same promoter control.

In order to obtain a more direct estimation of the relative efficiencies of the *Bacillus* β-glucanase and yeast α-factor signal peptides, a construct was made fusing the β-glucanase gene to the α-factor signal peptide under control of the ADH1 promoter. This fusion would be directly comparable to pJG317, differing only in the signal peptides used and to pJG314, where only the promoters differ. A summary of this new construct is given below and in Figure 3.8(a) to (c).

Plasmid pGS4, containing the α-factor signal peptide on a *Hin*dIII-*Bam*HI fragment was obtained from K.J.Shaw (Schering-Plough Research, N.J.). The signal contains the α-factor pre-pro region up to and including the KEX2 processing site, Lys-Arg (Shaw *et al.* 1988). To ease further manipulations, this fragment was ligated into the *Hin*dIII-*Bam*HI site of pUC19 (pUGS4) (Figure 3.8(a)). The *Stu*I site marks the location of the Lys-Arg cleavage site.

Figure 3.8(a). pUGS4 : pUC19 containing the α-factor signal.



The β-glucanase gene, including its complete signal peptide is present between two *Hin*dIII sites in pJG106 (Figure 3.8(b)). The *Dde*I site indicated with an asterix, marks the junction between the end of the *Bacillus* signal peptide and the beginning of the mature sequence of the gene (Murphy *et al.* 1984). The gene, minus its signal peptide was isolated from pJG106 on a *Dde*I fragment and the 5' overhangs were filled in to give a blunt ended fragment. This filled in fragment was then cut with *Hin*dIII and the β-glucanase gene, minus its signal peptide and pUC19 sequences, was purified from a gel. This blunt / sticky ended fragment was ligated (in frame) into *Stu*I/*Hin*dIII- cut pUGS4 to give pJGS4 (Figure 3.8(b)). The β-glucanase gene and α-factor signal peptide was removed from pJGS4 using *Bam*HI and ligated into pVT102U (Vernet *et al*, 1987), placing it under control of the ADHI promoter (Figure 3.8(c)). Orientation was confirmed by restriction digest and the plasmid named pVT314.

Figure 3.8(b). Construction of pJGS4 : pUGS4, with the β-glucanase gene fused to the α-factor signal peptide.

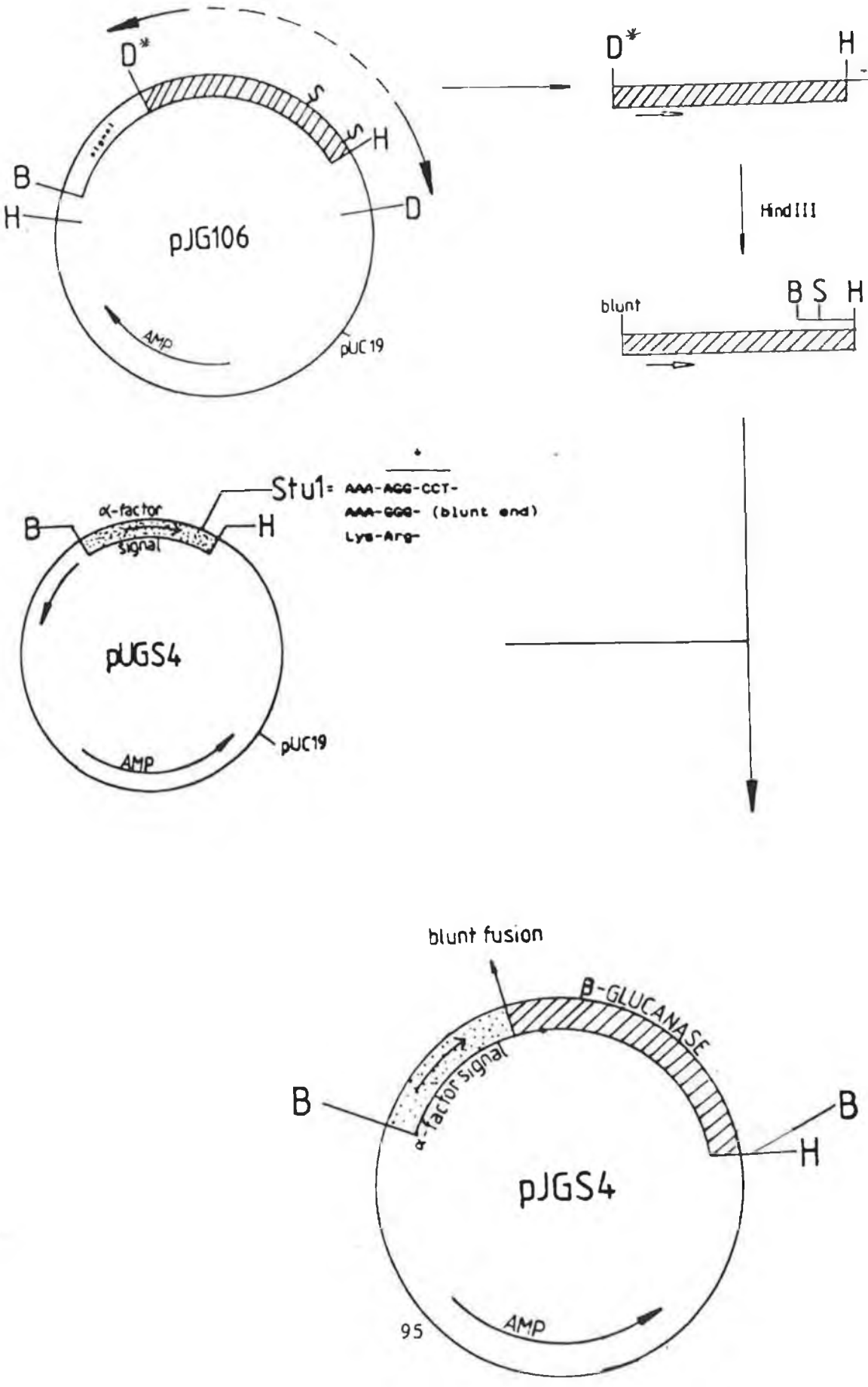
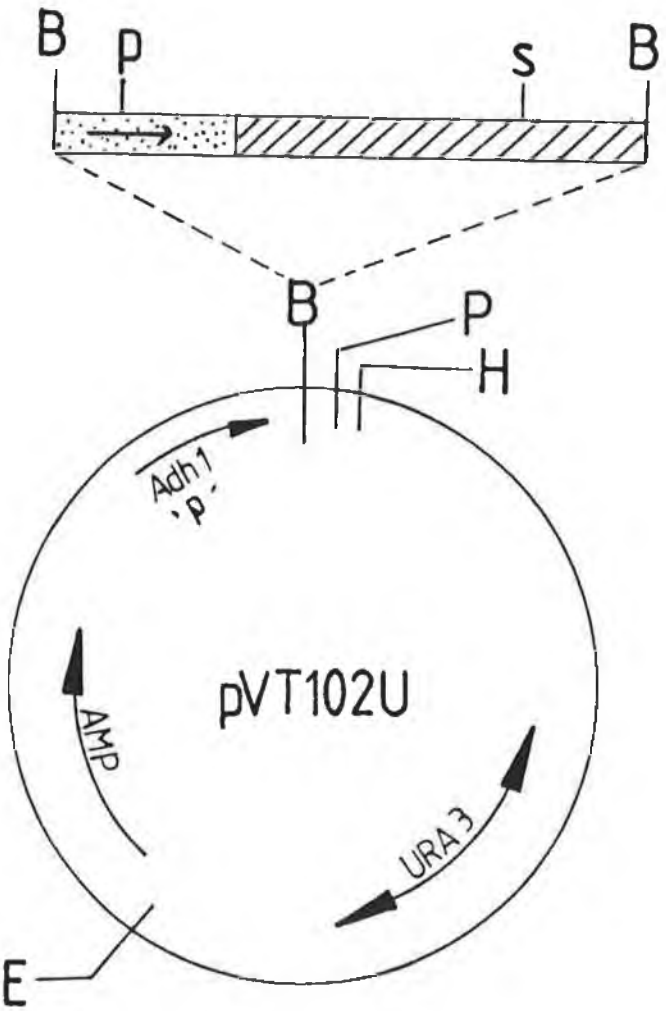


Figure 3.8(c). pVT314: β-glucanase gene under Adh1 promoter and α-factor signal peptide control.



α-factor signal =

β-glucanase gene =

pVT314 was transformed into DBY746 and expression and secretion of β-glucanase activity was monitored over a 54 hour growth period (Figure 3.9). Surprisingly, the levels of β-glucanase activity detected intracellularly and extracellularly in pVT314 were lower than in pJG317, despite the fact that both constructs contain the same promoter (Adh1), (see Fig.3.10 for comparison of extracellular enzyme activity). Intracellular levels of activity in pVT314 are marginally lower than those detected in pJG314 and over the majority of the growth cycle, extracellular levels are also lower. Only in stationary phase (at approximately 36hrs) do the secreted levels of β-glucanase in pVT314 approach those of pJG314.

Figure (3.9).

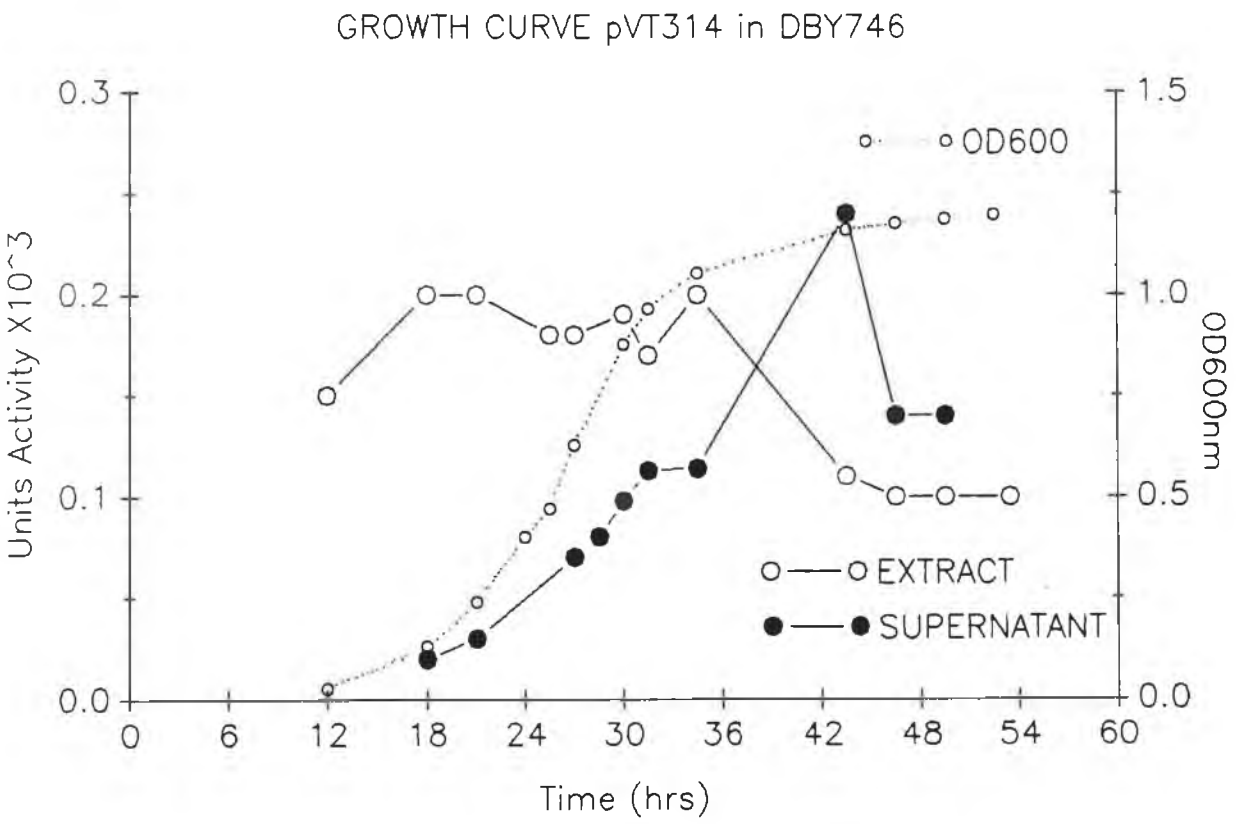


Figure (3.10) SECRETED β-GLUCANASE IN pJG317, pJG314 AND pVT314

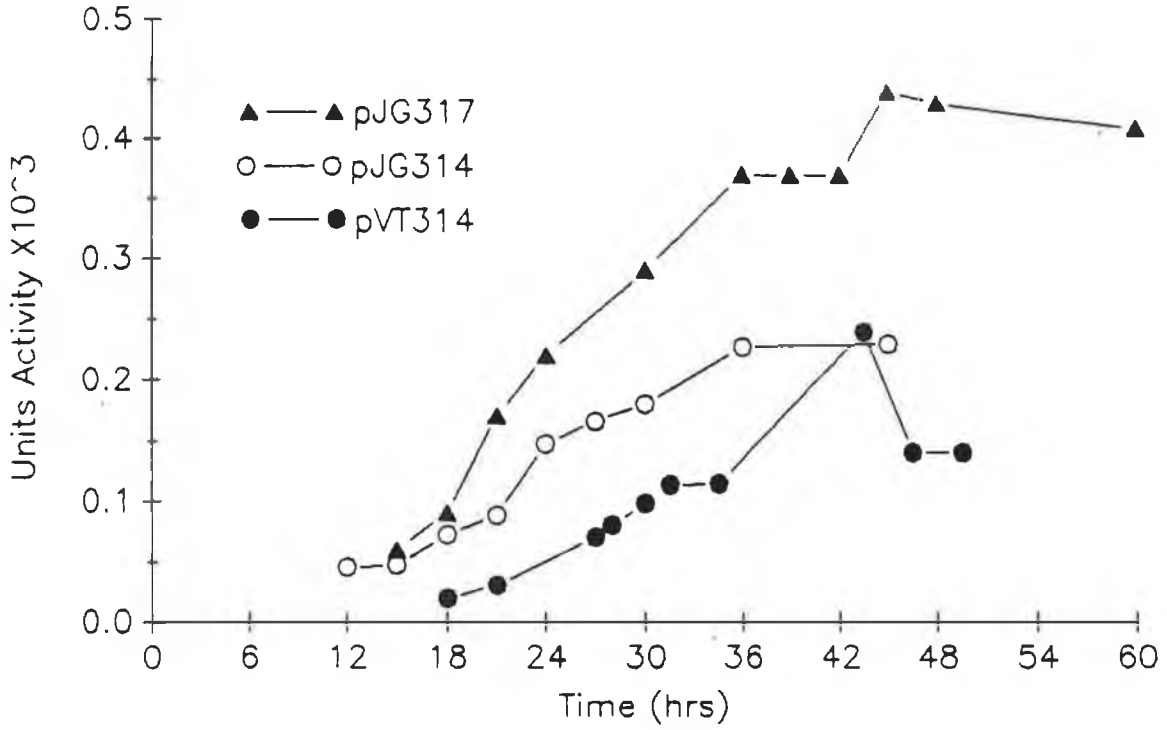


Table 3.1 summarizes the β-glucanase constructs and the levels of β-glucanase activity detected for each. To estimate the overall levels of activity in each case, intracellular and extracellular activity was taken at the point in the growth curve where the highest levels of enzyme are present extracellularly, ie. 42hrs for pJG314 and pVT314 and 45hrs for pJG317 and pJG205. Intracellular and extracellular β-glucanase were also expressed as percentages of the total activity at that point.

On one level, looking at the extracellular levels detected in the two bacterial signal constructs and the two α-factor signal constructs, this table shows that the bacterial signal peptide is capable of secreting up to 450 units of β-glucanase /ml while the α-factor signal secretes less (230-250 units/ml). Looking at the percentages, the figures also show that the two signals (excluding pJG205) are comparable in their efficiencies to secrete β-glucanase with 50-60% of the total activity detected extracellularly in each case.

Table (3.1). Summary of β-glucanase constructs and levels of enzyme production at one point in the growth cycle.

CONSTRUCT	PROMOTER	SIGNAL PEPTIDE
pJG205	ADH1	Truncated β-glucanase
pJG317	ADH1	Complete β-glucanase
pJG314	α-factor	α-factor
pVT314	ADH1	α-factor

Construct	Total Units Activity/ml	% Intracellular	% Extracellular
pJG205	3440	86%	14%
pJG317	820	46%	54%
pJG314	390	41%	59%
pVT314	370	38%	62%

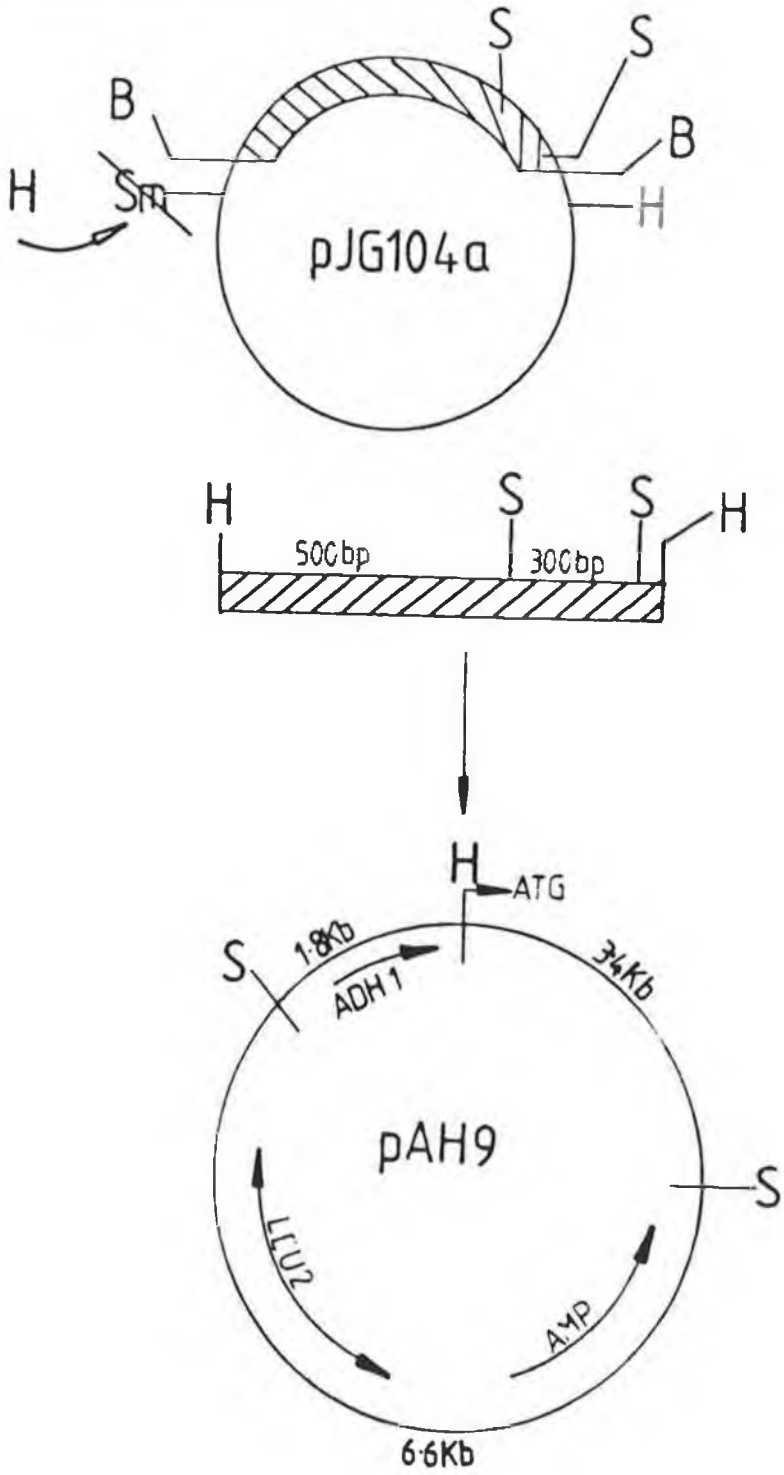
Note that this table represents β-glucanase activity at one point in the growth cycle only, in order to give some idea of the percentages of intracellular and extracellular production levels. The growth curves give a more general idea of the overall levels and characteristics of the strains, and as will become apparent, the situation is more complicated than this table suggests.

(3.6) **Removal of the β-glucanase signal peptide.**

The extracellular levels of β-glucanase activity observed in pJG317 and pJG205 would suggest that in yeast, this heterologous signal peptide is capable of directing β-glucanase out into the medium. If this bacterial signal peptide is to be useful as a general secretion signal for heterologous protein production in yeast, it is necessary to ensure that the extracellular β-glucanase levels observed in pJG317 and pJG205 are due to transport of the enzyme through the conventional secretory pathway and not due to cell leakage or lysis. In order to investigate this and to examine the overall production levels of the enzyme in these vector systems, it was decided to remove the β-glucanase signal peptide and express the gene directly under ADH1 promoter control. In this construct, the β-glucanase activity was expected to be limited to the yeast cytoplasm.

In removing the β-glucanase signal peptide, the ATG translational start site is also deleted. The pAH series of vectors (pAH9, pAH10 and pAH21) provide the ADH1 promoter on three different plasmids each with an ATG start site in one of the three reading frames and a *Hin*dIII cloning site (Ammerer, 1983). The β-glucanase gene, minus its signal peptide (starting from the *Dde*I site) was cloned into the *Bam*HI site of pUC8 after the addition of *Bam*HI linkers to the filled in ends to give pJG104, app.(4) (T. Ryan, D.C.U.). A *Hin*dIII linker (8'mer) was introduced into the *Sma*I site of pJG104 (pJG104a), (Figure 3.11). The *Hin*dIII fragment from pJG104a was transferred into the cloning site of pAH9 to generate an in-frame fusion between the ATG start and the β-glucanase gene. This construct was named pJBβ4. Orientation of the β-glucanase gene was confirmed by *Sal*I digests (Figure 3.12) and the plasmid (pJBβ4) transformed into DBY746. However, no β-glucanase activity was detectable intracellularly or extracellularly using the plate or DNS assay.

Figure (3.11). Construction of pJBβ4 : β-glucanase gene minus signal, under Adh1 promoter control.



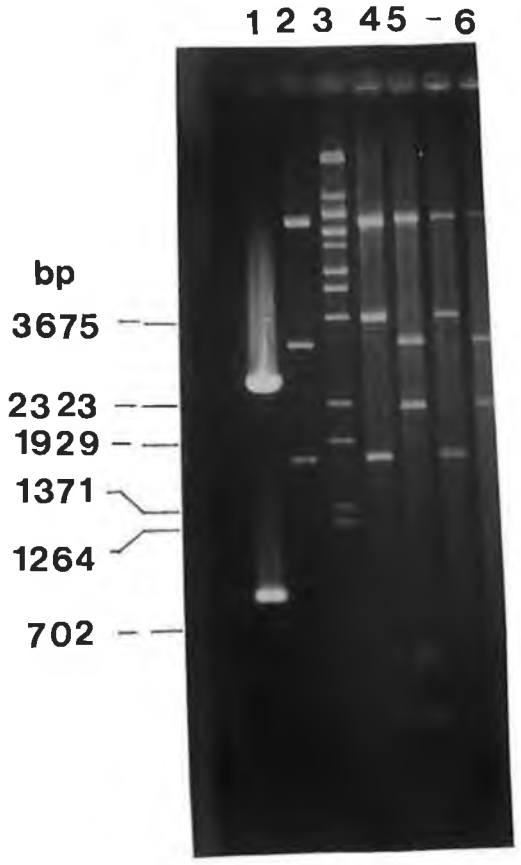
Sequence around ATG start of pJBβ4:

start of β-glucanase gene

ATG-TCT-CAA-/-AGC-TTG-GGG-GAT-CCG-TCA----------

pAH9 pJG104a

Figure (3.12). Restriction analysis of pJBβ4.
The approximate sizes of the restriction fragments in each digest are shown in brackets below. pAH9 with one copy of the β-glucanase gene (ie. pJBβ4) in the correct orientation is shown in lane 6.



Lane 1 : pJG106 / *Hin*dIII (2.6Kb; 0.9Kb).
Lane 2 : pAH9 / *Sal*1 / *Hin*dIII (6.6Kb; 3.4Kb; 1.8Kb).
Lane 3 : λ / *Bst*EII (relevant sizes marked on gel).
Lane 4 : pJBβ1 / *Sal*1 (6.6Kb; 3.8Kb; 1.8Kb; 0.3.Kb).
Lane 5 : pJBβ2 / *Sal*1 (6.6Kb; 3.4Kb; 2.3Kb; 0.5Kb; 03Kb : contains two copies of the β-glucanase gene).
Lane 6 : pJBβ4 / *Sal*1 (6.6Kb; 3.4Kb, 2.3Kb; 0.3Kb)

The pAH series of plasmids used in constructing pJBβ4, differ from pAAH5 in that they lack the ADH1 terminator sequence. Although the β-glucanase gene has its own terminator sequence present in all the plasmid constructs, it is possible that the bacterial terminator alone is not sufficient for effective termination. In the absence of a yeast terminator, transcriptional read through may have a destabilising effect on the β-glucanase transcript itself or on the plasmid as a whole. It was therefore decided to construct an alternative plasmid utilizing pAAH5 as a more direct comparison to pJG317 and pJG205. However, under certain conditions described at a later stage (section 3.9), pJBβ4 can produce active β-glucanase.

(3.7) **Construction of pJBC55A.**

An ATG start site was introduced in front of the β-glucanase gene in pJG104 via two intermediates: To create a suitable restriction sites for inserting the gene ultimately into pAAH5, a *Hin*dIII linker (8'mer), was fused to the filled in *Eco*RI site of pJG104 (pJG104b). This allows the β-glucanase gene to be isolated on a *Hin*dIII fragment. pJG104c was constructed from pJG104b by inserting a *Cla*I linker d(pCATCGATG), into the *Sma*I site (Figure 3.13). The proposed sequence around the 5' end of the gene is as follows:

start of β-glucanase gene
*

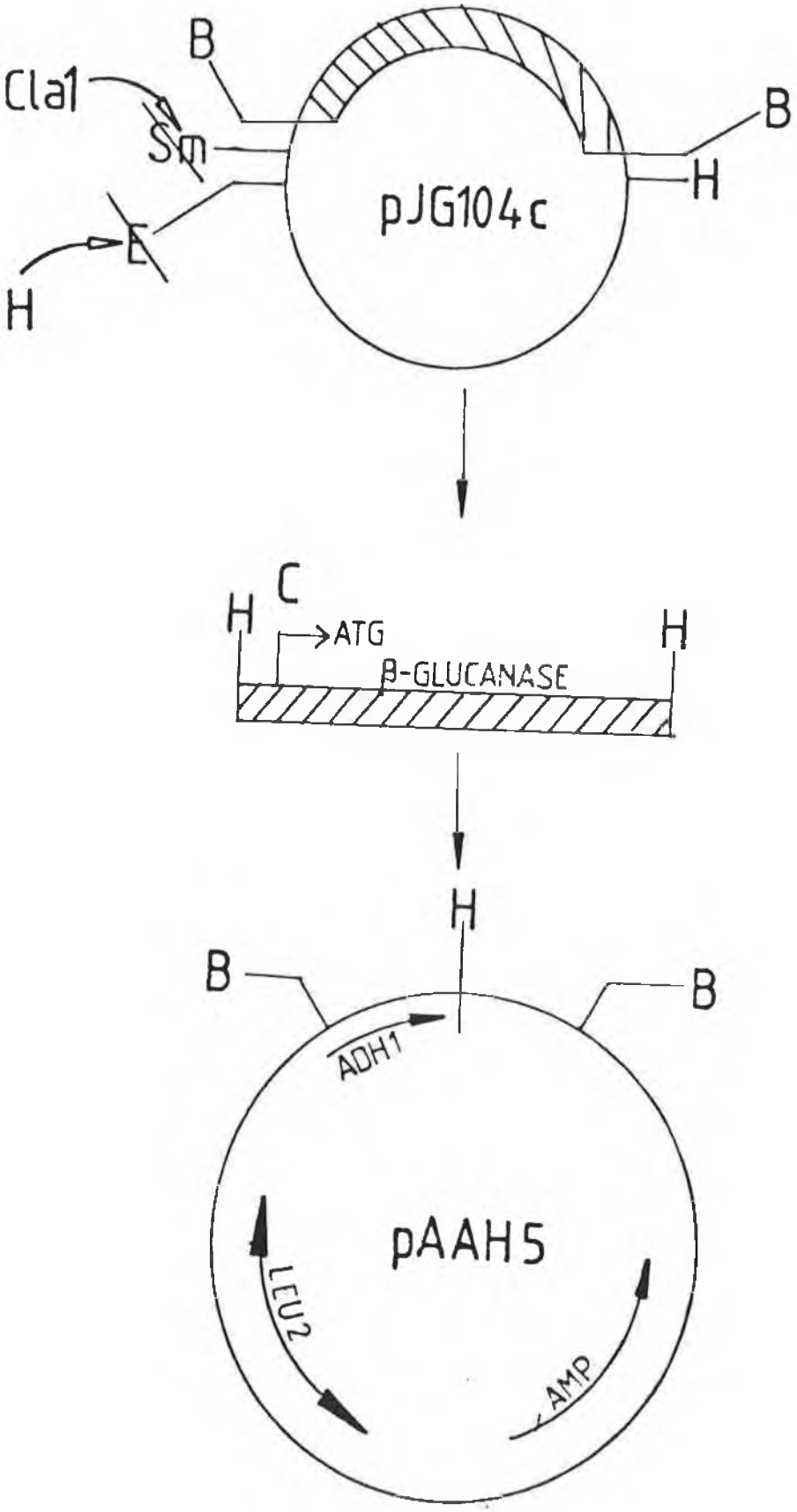
*Hin*dIII----CCCCATCGATG-GGG-GAT-CCT-TCA-GCT-.....-*Hin*dIII

*Cla*I linker — *Bam*HI linker

As a precaution, to ensure that the original pJG104 plasmid contained only one *Bam*HI linker as indicated, pJG104c was restricted exhaustively with *Bam*HI, the vector and insert fragments gel purified and ligated to reform pJG104c. The resultant plasmid was then cut with *Cla*I, gel purified and religated to ensure that only one *Cla*I linker was also present. The β-glucanase gene was isolated using *Hin*dIII and ligated into the *Hin*dIII site of pAAH5 (pJBC55A), (Figure 3.13). In

DBY746, like pJBβ4, pJBC55A produced no detectable β-glucanase activity.

Figure (3.13). Construction of pJBC55A.



(3.8) **Northern analysis of pJBβ4 and pJBC55A transcripts.**

To investigate if specific β-glucanase transcripts could be detected in pJBβ4 and pJBC55A and to gain some insight into the relative stabilities of these transcripts compared to pJG205 and pJG317, Northern analysis was carried out. Total RNA was prepared from all of the strains containing the ADH1 constructs: pAAH5, pJG317, pJG205, pJBC55A, pJBβ4, pAH10β and pAH21β. pAH10β and pAH21β contain the β-glucanase gene cloned into the *Hin*DIII site of pAH10 and pAH21 (constructed by M.Duffy, D.C.U.). Untransformed DBY746 was also included as a negative control.

The RNA was fractionated by formaldehyde gel electrophoresis (2.26), and blotted bi-directionally onto nitrocellulose. One filter was hybridised to a β-glucanase probe (the *Hin*dIII fragment from pJG106) and the second to the yeast β-actin gene (from pRB155, Shortle *et al*, 1982). The actin probe was included as an internal control to equate hybridisation intensities. Figures 3.14(a) and (b) show the RNA gel and corresponding β-glucanase-probed autoradiograph. Three transcripts were detected using the β-glucanase probe corresponding to pJBC55A (approximately 1480bp), pJG205 (approximately 1520bp) and pJG317 (approximately 1620bp). No transcript was detected in pJBβ4 or in the pAH control plasmids.

Results from densiometric scanning of the β-actin and β-glucanase transcripts are shown in Table (3.2). The relative intensities of the β-actin and β-glucanase transcripts are given. Correcting the β-glucanase values for the amount of RNA loaded in each case (guaged from the β-actin reading), shows that the relative intensities of the β-glucanase specific transcripts in pJBC55A, pJG205 and pJG317 are 2.5 : 0.7 : 0.8 respectively. This indicates that there is up to three times the amount of β-glucanase specific transcript in the signal-less construct compared to the other two. The relative intensities of pJG205 and pJG317 are approximately the same.

Figure 3.14(a). Northern gel showing RNA preparations from *S.cerevisiae* strains.

Lane 1: *E.coli* ribosomal RNA (23S and 16S).

Lane 2: DBY746, negative control.

Lane 3: pJBC55A.

Lane 4: pJBβ4

Lane 5: pJG205.

Lane 6: pJG317.

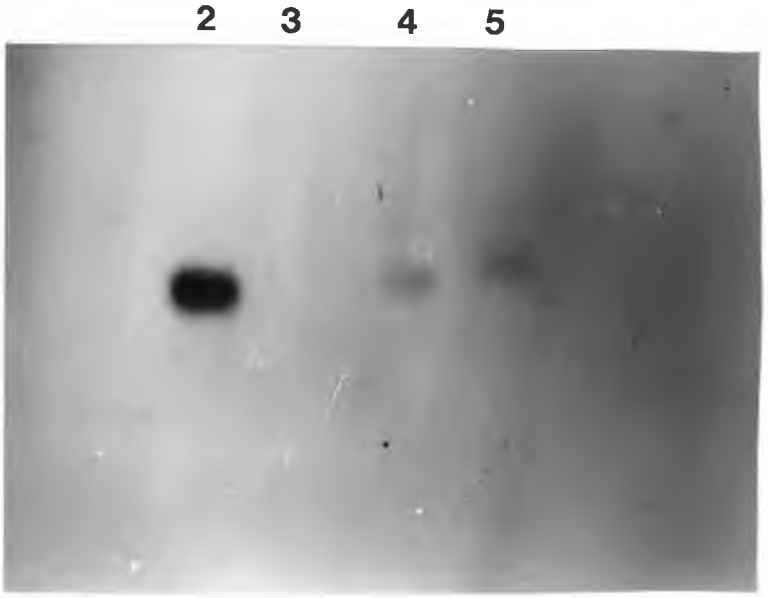
Lane 7: pAAH5, negative control.

Lane 8: *E.coli* ribosomal RNA (23S and 16S).

Lane 9: pAH10β.

Lane 10:pAH21β.

Figure 3.14(b). Autoradiograph of β-glucanase transcripts.



Lane 2: pJBC55A; Lane 3: pJBβ4; Lane 4: pJG205; Lane 5: pJG317.

Table (3.2).
Relative intensities of transcripts calculated by densiomtric scanning.

	pJBC55A	pJG205	pJG317
β-ACTIN	2.5	1.5	1.0
β-GLUCANASE	6.25	1.0	1.25
CORRECTED β-GLUCANASE	2.5	0.7	0.8

(3.9) **Assay of pJBβ4 and pJBC55A in a pep4-3 (protease deficient) mutant of *S.cerevisiae*.**

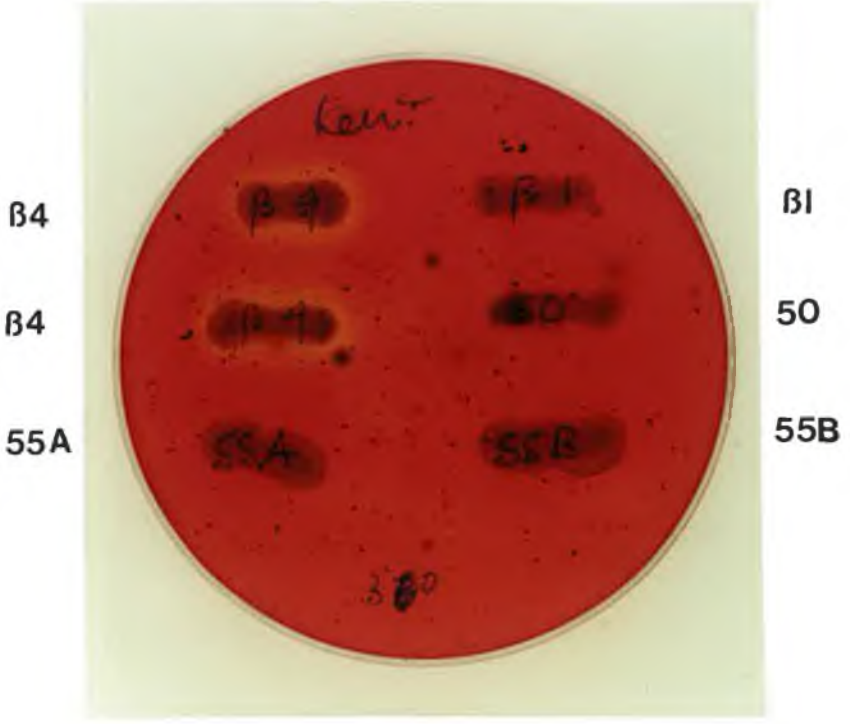
With the presence of a stable transcript in pJB55A, the possibility remained open that the lack of detectable enzyme activity associated with this construct, may be a result of instability of the heterologous protein in the yeast cytoplasm. In the cytoplasm, β-glucanase could be a target of cellular proteases *in vivo*, possibly even a substrate of the ubiquitin directed degradation system (section 1.1.2). Folding differences, due to the absence of the signal peptide sequence may also contribute to any instability of the β-glucanase enzyme in the cytoplasm. In addition, the protein may be susceptible to the action of vacuolar and other proteases, which can be released during assaying procedures (see section 1.1.2). To investigate this, the signal peptide-minus constructs were transformed into the protease deficient mutant MD50. This mutant is deficient in the four major vacuolar proteinases PrA1, PrB1, PrC1 and CpS1 and contains the *pep*4-3 mutation (Ammerer *et al* 1986).

pJBC55A and pJBβ4 were transformed into MD50 and plated on minimal medium. The cells were incubated at 25^{0}C for 48hrs, lysed using a hot agar overlay (section 2.18) and incubated at 25^{0}C for a further 48hrs before staining with congo red dye. The lower temperature was chosen to minimize proteolysis, enzyme instability and temperature activation of proteases after lysis. Staining with congo red revealed halos around pJBβ4 (plated in duplicate), indicating intracellular β-glucanase activity (Figure 3.15). The halo observed for pJBβ4 was considerably smaller than those generated around pJG317 and pJG205 (also transformed into the MD50 mutant), presumably reflecting differing levels of active enzyme. The apparent instability of the pJBβ4 RNA transcript (section 3.8), must compound this protease problem and the combination of these two factors are likely to be the reason for the lack of detectable activity for pJBβ4 in DBY746.

In contrast to pJBβ4, no β-glucanase activity was detected in pJBC55A transformed into MD50, despite the obvious stability of

the RNA transcript. Possible reasons for this, including potential problems at a translational level are discussed in section (4.2).

Figure (3.15).



β-glucanase production in MD50 transformed with pJBC55A, pJBβ4 and the negative control pJBβ1 (pAH9 containing the β-glucanase gene in the opposite orientation to pJBβ4). pJBC50 and pJBC55B are identical independant isolates of pJBC55A.

(3.10) Analysis of β-glucanase secretion in secretion defective mutants.

At the non-permissive temperature (37°C), secretion defective (*sec*) mutants of *S.cerevisiae* block secretion at various stages along the secretory pathway. The ability of the bacterial signal peptide to transport the β-glucanase enzyme through the conventional yeast secretory pathway was examined in a series of these mutants. Table (3.3) summarizes the *sec* strains used and the characteristics of each mutant.

Table (3.3). Summary of *sec* mutants.

Strain	Mutation	Block In Pathway
RSY11	*sec*18	ER to Golgi, (lacks protein involved in vesicle fusion).
RSY12	*sec*53	ER, (glycosylation mutant, section 1.6)
RSY45	*sec*1	Secretory vesicles between Golgi and cell surface.

pJG317 was transformed into each of the mutant strains and β-glucanase secretion examined at permissive and non-permissive temperatures, using the following procedure:

Mutants, transformed with pJG317, were grown to OD_{600}=1.0, at 25°C. The cells were washed twice in 1M sorbitol to remove β-glucanase present in the medium and divided into two equal samples. The samples were resuspended in fresh medium prewarmed to (a) 25°C and (b) 37°C. Growth was allowed to continue at these respective temperatures for 3-6 hours. The cells were then harvested and the extracts and supernatants assayed for β-glucanase activity using the plate assay. 6mm wells were cut into buffered agar containing 0.05% lichenan. Supernatants were added to the wells and the plates incubated at 37°C overnight. Halos indicating β-glucanase activity were visualised by staining with congo red dye (section 2.18) and Figure (3.16). As a control, this procedure was also carried out with DBY746 transformed with pJG317.

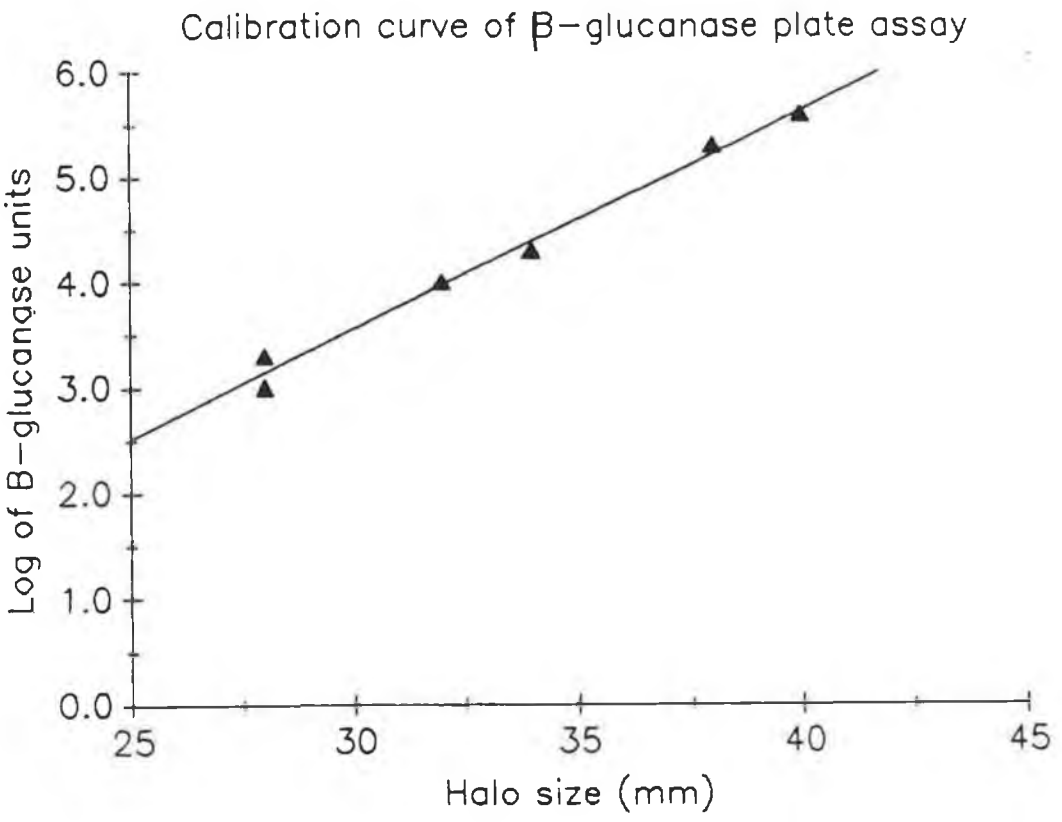
A block in secretion of β-glucanase was observed in both RSY11

and RSY45 at the restrictive temperature (Fig.3.17). This indicates that the bacterial signal peptide is acting as a secretion signal in its heterologous host.

In RSY12 (*sec*53), an unexpected result was observed. In this mutant, no block in secretion of β-glucanase was seen at the non-permissive temperature. Instead, a significant increase in the level of enzyme activity was detected at 37^{0}C compared to 25^{0}C (Fig.3.18). More accurate quantitaton of this increase, using the DNS assay, indicates that at the restrictive temperature, up to 3 times as much activity is produced both intracellularly and extracellularly.

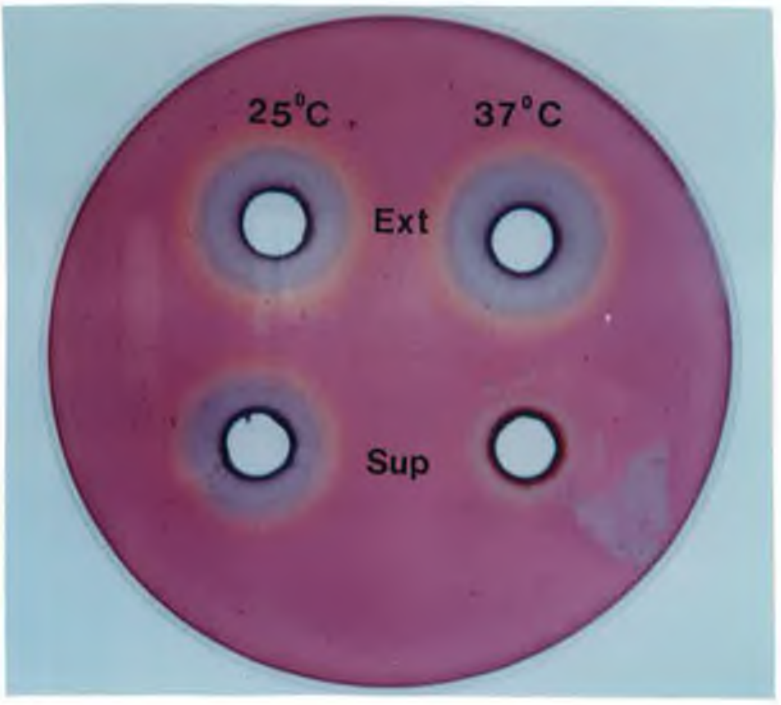
pJG205 was also tested for β-glucanase activity in these *sec* mutant strains. A block in secretion of the enzyme was observed in RSY11 and RSY45 at the non-permissive temperature. Similar to pJG317, an increase in β-glucanase activity was detected both intracellularly and extracellularly, at the restrictive temperature in RSY12.

Figure (3.16)

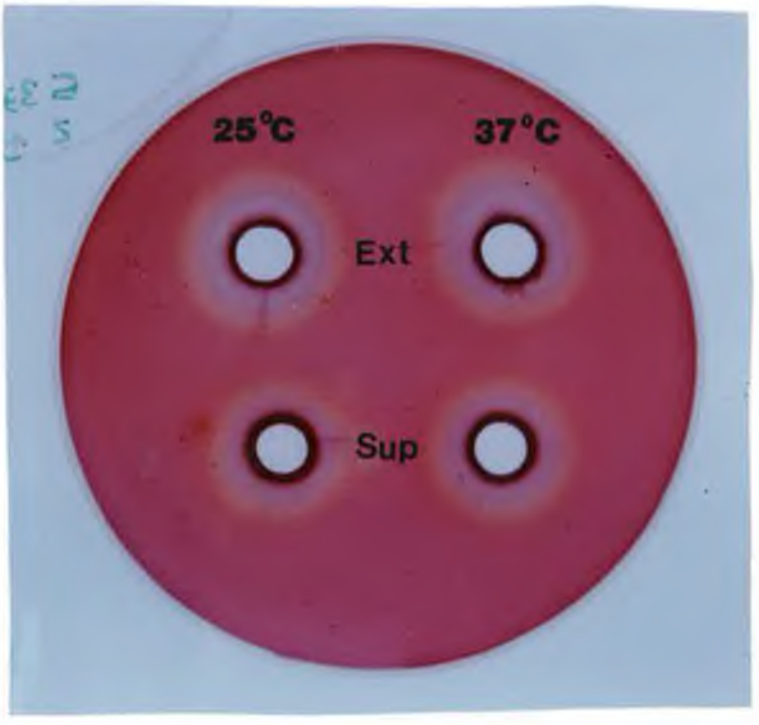


Calibration curve for the β-glucanase plate assay showing that an increase in halo size can be related to increased levels of activity and is therefore useful for qualitative assessment of assay results.

Figure (3.17).

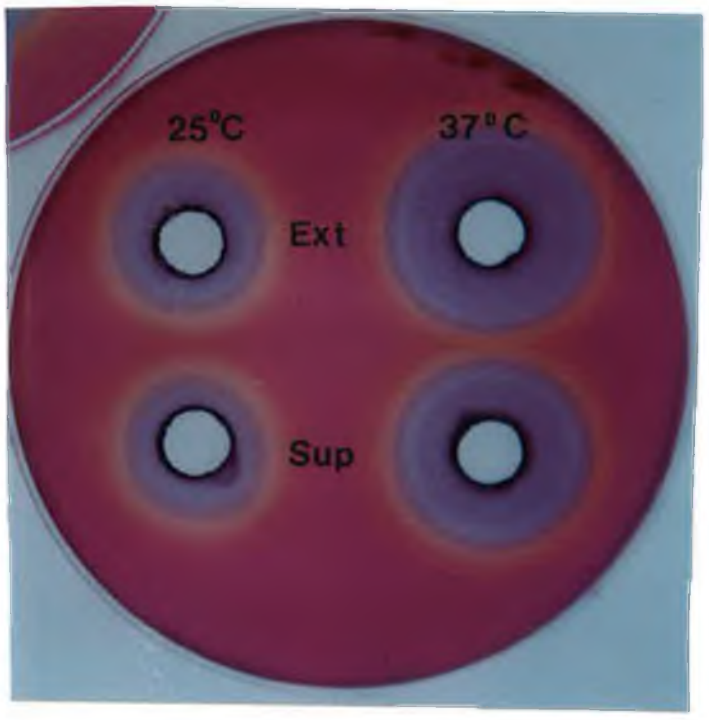


(a) Block in secretion of β-glucanase in RSY11 (pJG317) at 37°C. A similar block is seen with RSY45 (not shown).

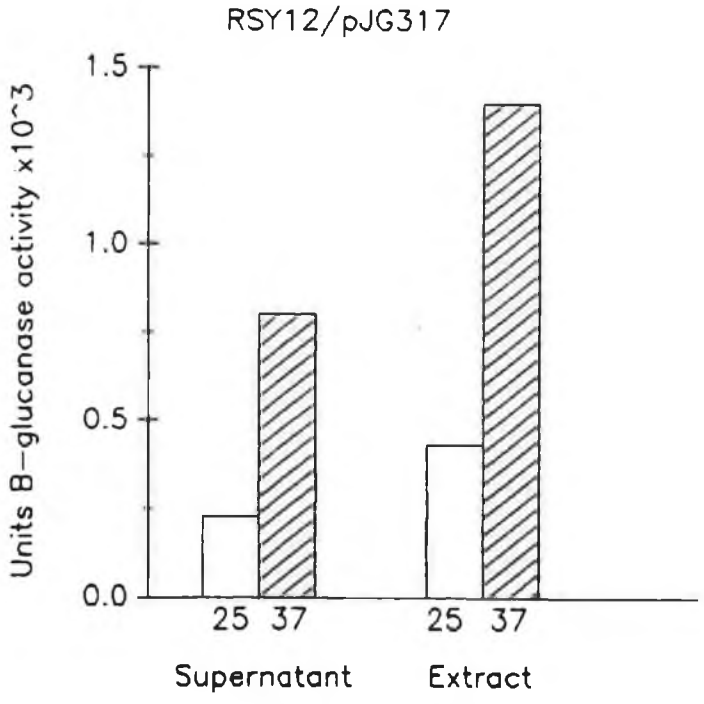


(b) DBY746 control assay (no block in secretion in wild type).

Figure (3.18).



Increase in β-glucanase activity seen at 37^0C in RSY12 (pJG317) at intracellular and extracellular levels.

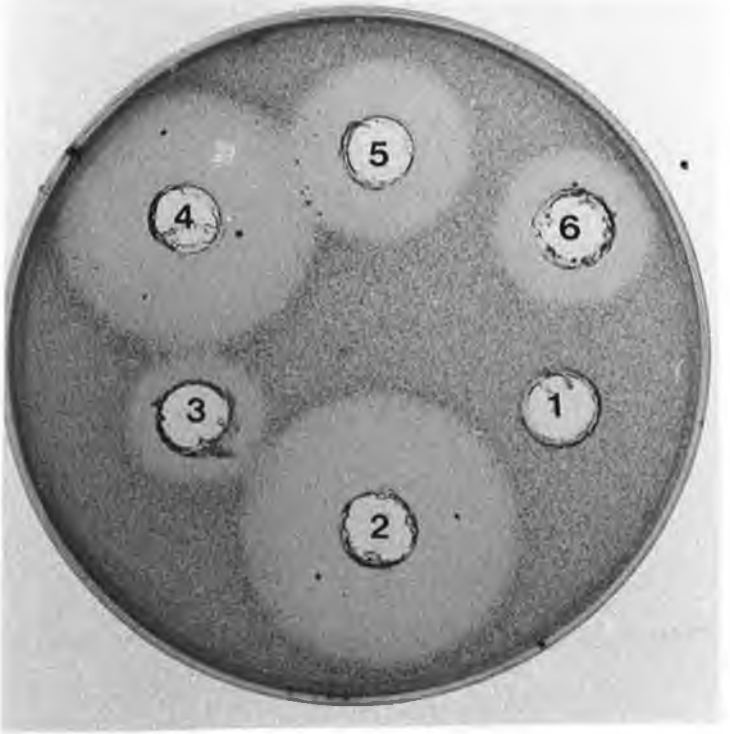


DNS assay showing the extent of the increase in RSY12 (pJG317).

To ensure that the mutant phenotype was being induced in RSY12 in the presence of the β-glucanase plasmids, an assay was adapted to assess the secretion patterns of the homologous α-factor protein in this mutant strain:

α-factor pheromone inhibits the growth of strains of the opposite mating type (Mat-a). RC631 is a Mat-a strain containing the *sst*2-1 mutation, rendering it extrasensitive to the inhibitory effects of α-factor (Julius *et al.* 1983). RC631 was incorporated into MYGP agar as described in section 2.32. 6mm wells were cut in the agar and supernatants from Mat-α strains added to the wells (RSY11,12 and 45 and DBY746 are all Mat-α). The plates were incubated at 30^{0}C for 48hrs to allow growth (Fig.3.19). Clear halos around the wells show the growth inhibition of RC631 due to the presence of α-factor in the supernatants of the Mat-α cells. The large halos in wells 2 and 4 are due to overexpression of α-factor in cells carrying the plasmid p69A.

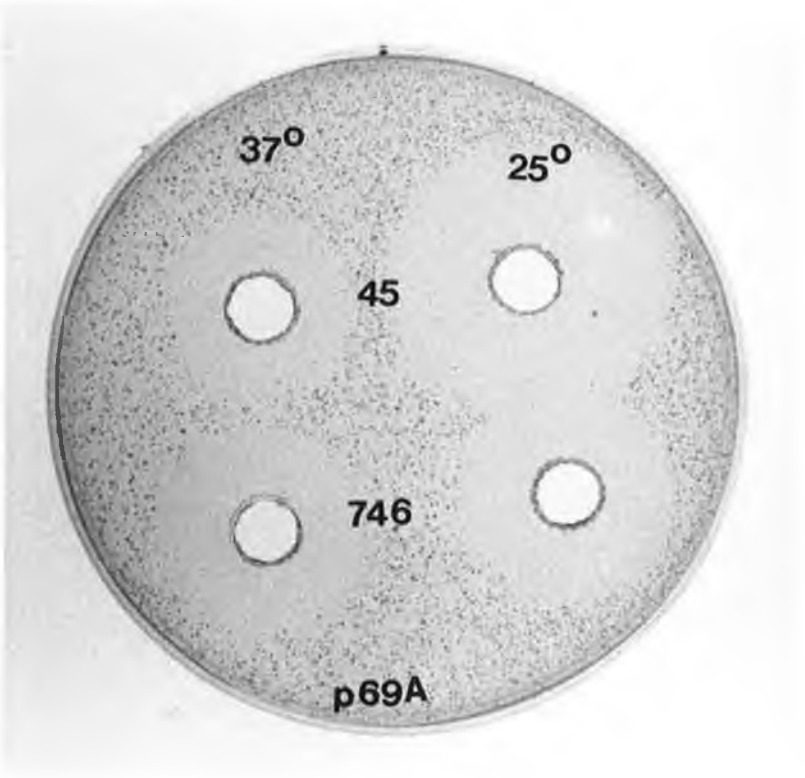
Figure (3.19). The α-factor plate assay



Well 1: RC631(Mat a)
Well 2: RSY45/p69A
Well 3: RSY45
Well 4: DBY746/p69A
Well 5: DBY746
Well 6: RSY12

p69A (app.2), is a multicopy plasmid containing the α-factor structural genes under control of the α-factor promoter and signal peptide (Kurjan and Herskowitz 1982). When the mutant phenotype is induced in mutants RSY11,12 and 45, carrying this plasmid, a block in secretion of α-factor is observed at 37°C (Fig. 3.20). This block is reflected in a decrease in the zone of inhibition of RC631.

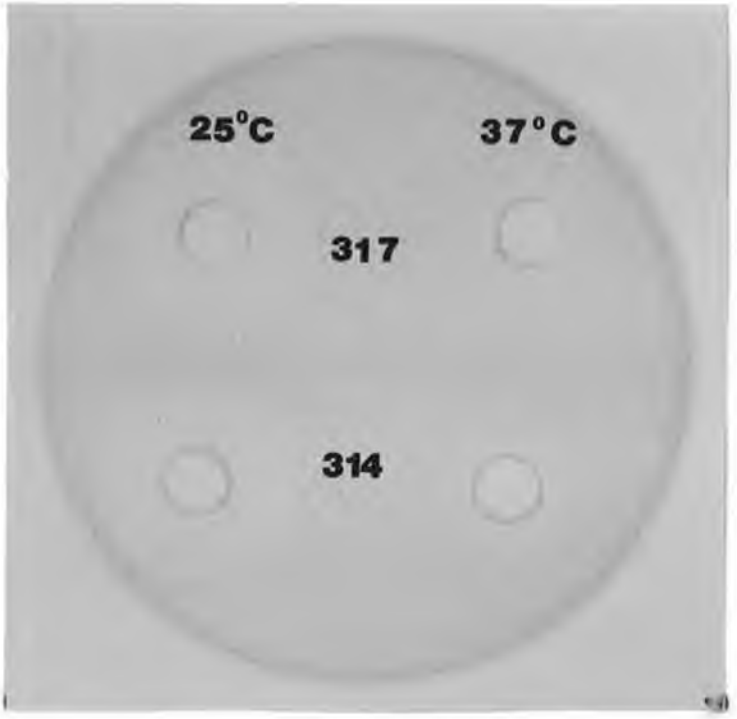
Figure (3.20).



Block in α-factor secretion at 37°C in RSY45 transformed with p69A. Wild type DBY746 shows no block at that temperature.

The *sec* mutant strains, transformed with the β-glucanase plasmids, also exhibit a block in secretion of endogenous levels of α-factor at 37°C (Fig.3.21). This confirms that the increase in β-glucanase activity detected in RSY12 at the non-permissive temperature, does not interfere with the normal secretion patterns of the cell and that the abnormal behavior is associated only with the heterologous gene product.

Figure (3.21)



Block in α-factor secretion at 37°C in supernatants of RSY12 containing pJG317 and pJG314.

To investigate if the increase observed in pJG317 and pJG205 was associated with the *Bacillus* signal peptide, pJG314, which contains the β-glucanase gene under control of the α-factor signal peptide, was also tested for secretion of β-glucanase at the restrictive temperature in RSY12. An increase was observed both intracellularly and extracellularly in this case also. Because pJG314 contains the yeast α-factor signal peptide, the increase in activity cannot be signal specific but must be associated with the β-glucanase enzyme itself. To investigate whether this effect is peculiar to β-glucanase, another heterologous protein, a *Bacillus* α-amylase, was also tested in RSY12 for secretion at the non-permissive temperature. Secretion of this heterologous enzyme was blocked at 37°C (data not shown).

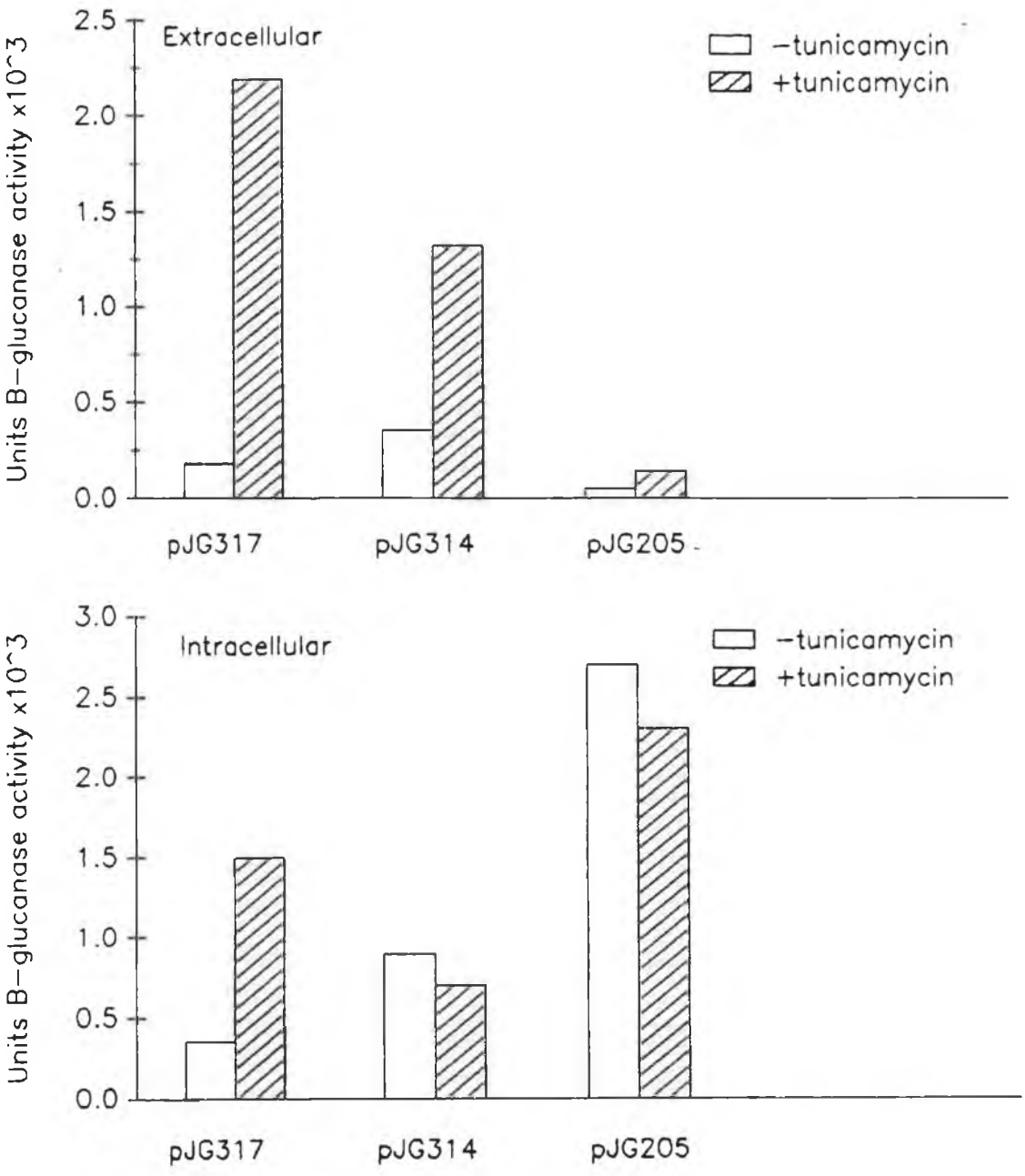
(3.11) **The effect of tunicamycin on β-glucanase production.**

RSY12 (*sec*53) is deficient the phosphomannomutase activity responsible for the conversion of mannose-6-phosphate to mannose-1-phosphate (section 1.5.1). The generation of these mannose residues is required for the assembly of core oligosaccharide units in the N-glycosylation groups of secretory proteins. At 37^{0}C, *sec*53 mutants accumulate secretory proteins with incomplete core oligosaccharide units in the ER (section 1.5.1). Tunicamycin also inhibits the assembly of these core units, although at an earlier stage in their synthesis ie. the addition of the first GlcNAc residue.

Sequence analysis of the β-glucanase gene shows that the enzyme has four potential glycosylation sites, at positions 31,38,64 and 185 (app.1). The result obtained with RSY12 suggest that the increase in activity might be related to the glycosylation pattern of the heterologous protein in yeast. If this is so, tunicamycin treatment of wild type cells carrying the β-glucanase plasmids should yield similar results. This possibility was tested for pJG317, pJG205 and pJG314 in DBY746: Cells were grown to OD_{600}=1.0 at 30^{0}C, harvested, washed twice in 1M sorbitol and divided into two equal samples. Tunicamycin (10μg/ml) was added to one sample in each case and both samples returned to 30^{0}C for 6hrs. Supernatants were assayed for β-glucanase activity using both the well plate and DNS assays. Treatment with tunicamycin resulted in an increase in β-glucanase activity in the supernatants of all three strains (Fig.3.22, for results of the DNS assay). In pJG317, the effect was more striking than observed in the *sec*53 mutant, with almost 9 times the activity being released in this instance with the addition of tunicamycin. In pJG205 and pJG314 an increase of 3-4 fold was observed.

Intracellularly, pJG317 also exhibits an increase in activity after treatment with tunicamycin. This is in contrast to pJG205 and pJG314 where lower level of activity are detected inside the cell.

Figure (3.22) Extracellular and intracellular β-glucanase activity in pJG317, pJG314 and pJG205, after treatment with tunicamycin.



The results obtained with tunicamycin treatment correlate well with the observations in *sec*53, in that inhibiting glycosylation causes an increase in β-glucanase activity in both cases. Each of these treatments causes disruptions to core oligosaccharides addition, an event that occurs in the ER.

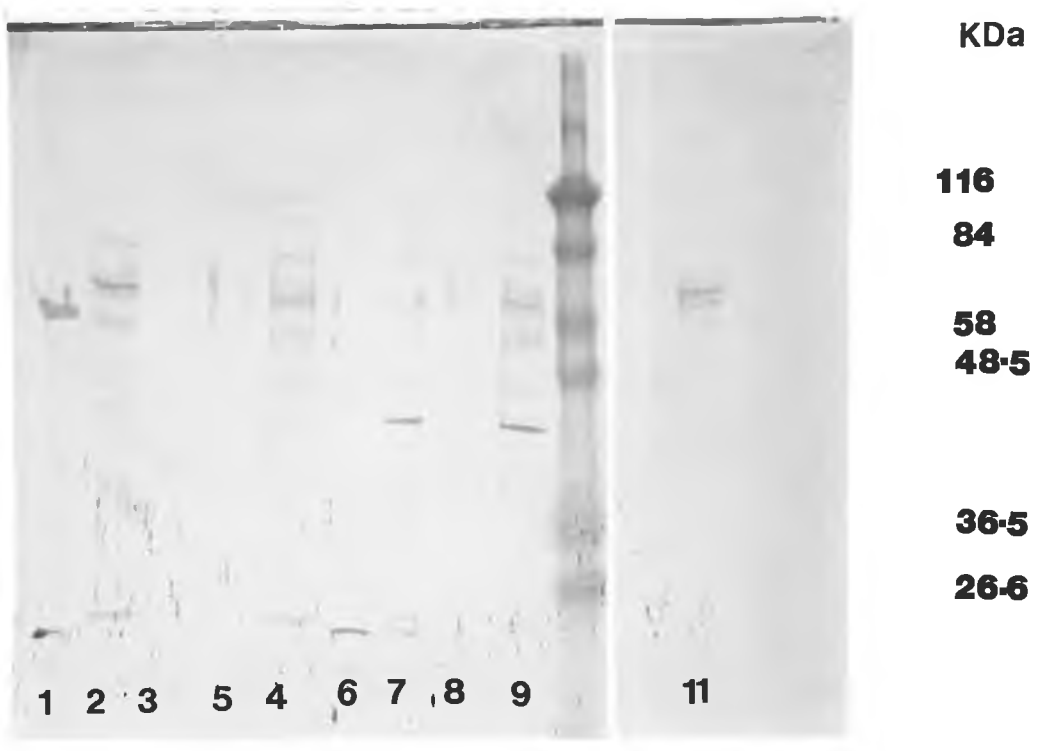
(3.12) Western blot analysis of the *Bacillus* β-glucanase enzyme

To examine the glycosylation pattern of the *Bacillus* enzyme in DBY746, Western blot analysis was carried out. The β-glucanase antibody used in this analysis was obtained from E.Gormley, T.C.D. This polyclonal antibody was raised against a commercial preparation of the mature *B.subtilis* β-glucanase enzyme (E.Gormley, Ph.D. thesis, T.C.D.).

Extracts and supernatants were prepared (section 3.11) from pJG317, pJG205 and pJG314 after growth in the presence and absence of tunicamycin. The samples were prepared for SDS PAGE as described section (2.22). The gels (10%) were blotted onto nitrocellulose and hybridised to the β-glucanase antibody and enzyme-conjugated secondary antibody (section 2.23). Figures (3.23) and (3.24) show the Western blots of extracts and supernatants from pJG317 pJG314 and pJG205.

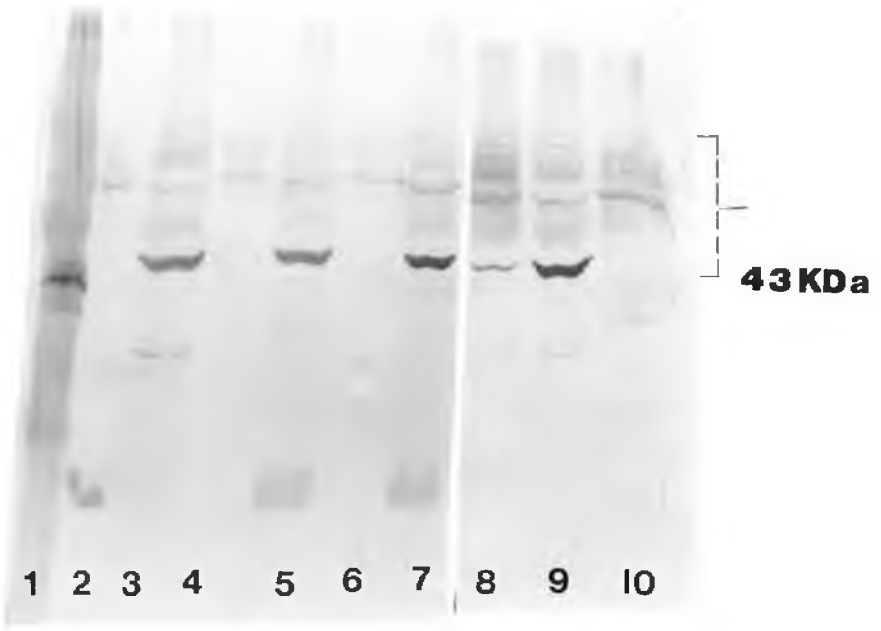
Figure (3.23) and (3.24). Western blots showing extracts and supernatants prepared from pJG317, pJG314 and pJG205, hybridised to the β-glucanase antibody..

Figure (3.23).



Lane 1: *B.subtilis*, control
Lane 2: pJG314 (Ext) + Tunic.
Lane 3: " (Sup) + Tunic.
Lane 4: " (Ext) - Tunic.
Lane 5: " (Sup) - Tunic.
Lane 6: pJG317 (Sup) + Tunic.
Lane 7: " (Ext) + Tunic.
Lane 8: " (Sup) - Tunic.
Lane 9: " (Ext) - Tunic.
Lane 10: Sigma prestained markers.
Lane 11: DBY746 (Ext).

Figure (3.24).



Lane 1: marker.
Lane 2: *B.subtilis*, control.
Lane 3: pAAH5 (Ext)(control).
Lane 4: pJG205 (Sup) - Tunic.
Lane 5: " (Ext) - Tunic.
Lane 6: pJG205 (Sup) + Tunic.
Lane 7: " (Ext) + Tunic.
Lane 8: p69B (Ext) control.
Lane 9: pAAH5 (Ext) control.
Lane 10: DBY746 (Ext) control.

These Western blots reveal no high molecular weight forms that are specific for β-glucanase, which would indicate that glycosylated enzyme is present. Figure (3.23), lane 9, shows the extract from pJG317 cells. Two bands of molecular weights 22KDa and 24KDa are present. The mobility of the lower band corresponds exactly to the 22KDa *Bacillus* commercial enzyme preparation (lane 1) and represents fully processed, unglycosylated β-glucanase. The higher molecular weight band (24KDa) corresponds in size to that expected for the unprocessed unglycosylated form of the enzyme with the signal peptide still intact. In pJG205 (Fig.3.24, lane 5), two bands are also visible, running very close together and corresponding to processed and unprocessed forms of unglycosylated β-glucanase. In this case, the unprocessed band contains the truncated form of the β-glucanase signal and this can account for it running very close to the processed band in this sample. In pJG314 (Fig. 3.23, lane 4), the β-glucanase band observed in the extract, is larger than the commercial control. This slightly slower mobility can be explained by retention of the Glu-Ala repeat sequences on the β-glucanase N-terminus. These sequences are present after the Lys-Arg cleavage site in the α-factor signal peptide that was used in this construction and are normally removed by the action of the STE13 gene product (section 1.5.4) (see section 4.6 for details).

A 43KDa band is present in extracts from pJG317, pJG314 and pJG205. This band also appears in extracts prepared from DBY746 carrying the vectors used to construct these plasmids, pAAH5 and p69B (lanes 9 and 10, Fig. 3.24), and is therefore not associated with β-glucanase activity in these strains. The other high molecular weight bands, indicated with the square bracket, are also present in these lanes and are assumed to be due to non specific hybridisation of the antibody preparation.

In the absence of tunicamycin, no bands are visible in the supernatant fractions corresponding to each of the above extracts. Although these samples contain active β-glucanase, even increased concentrations of supernatant added to each lane reveals no evidence of glycosylated or unglycosylated bands

(data not shown).

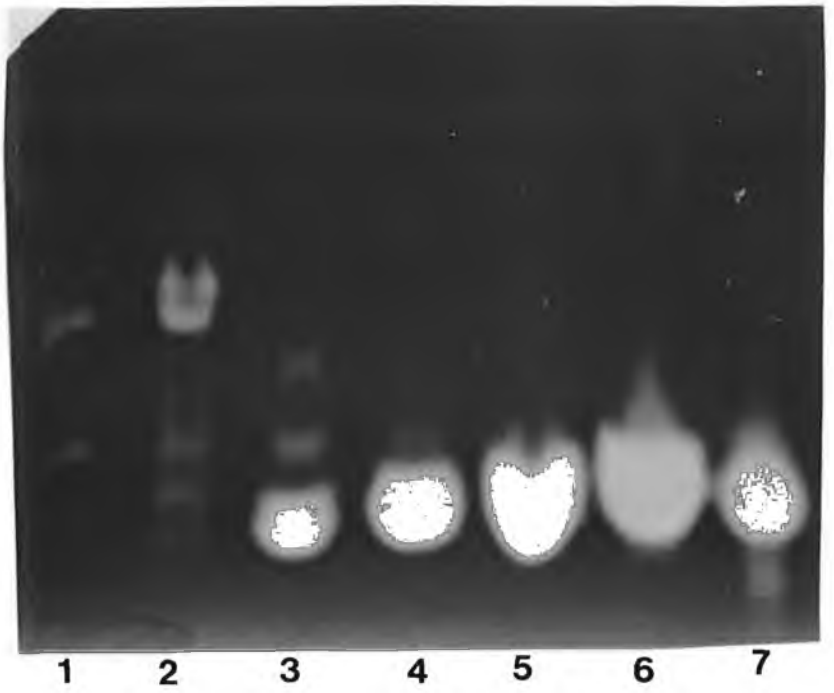
The increase in β-glucanase activity, seen previously after tunicamycin treatment (section 3.11), is reflected in the Western blot with the appearance of a strong band at 22KDa in the supernatant fraction of pJG317 (Fig.3.23, lane 6). The band corresponds to fully processed, unglycosylated β-glucanase. This finding supports the idea that some form of glycosylation of the enzyme must be taking place. It is possible that the glycosylated form is secreted but is only recognised by the antibody when the addition of the sugar moieties is inhibited by the action of tunicamycin. Glycosylated forms of β-glucanase are not detected in cell extracts from pJG317 using the β-glucanase antibody. However, the increase in intensity of the two β-glucanase specific bands, after tunicamycin treatment, suggests that glycosylated forms are also present intracellularly at this stage. In pJG314 and pJG205 extracellular fractions, no bands appear in the supernatants of either strain even after tunicamycin treatment.

(3.13) **Activity gel analysis of the β-glucanase enzyme.**

The Western blot data suggests that much of the intracellular pool of β-glucanase is fully processed with respect to signal peptide cleavage. Extracellular fractions, although containing β-glucanase activity (indicated by DNS and plate assays), reveal no bands corresponding to the enzyme. The appearance of a processed, unglycosylated band after treatment with tunicamycin, suggests that some level of glycosylation must be occurring, at least in the secreted enzyme fraction and it is possible that the problem lies with the antibody. This antibody, raised against the bacterial form of the enzyme, may be unable to recognise modified forms of the enzyme produced in yeast.

To address this problem in a more direct manner, activity gel analysis was carried out. It was hoped that by taking this approach that any higher molecular weight forms of the enzyme, if active, would be detected. The procedure for this zymogram-type gel analysis is outlined in detail in section (2.24). Bands representing active β-glucanase appear as clearings on a congo red stained background. Figure (3.25) shows a 10% polyacrylamide activity gel with extract samples of pJG317, pJG314 and pJG205 prepared at mid-log and stationary phases of the growth cycle. In contrast to the Western analysis, higher molecular weight forms, that did not react with the β-glucanase antibody, can be clearly seen. This indicates that the enzyme is indeed glycosylated and that these forms of the enzyme have activity.

Figure (3.25). Activity gel showing intracellular β-glucanase activity in pJG314, pJG317 and pJG205.



Lane 1 : pJG314 early
Lane 2 : " late
Lane 3 : pJG317 early
Lane 4 : " late
Lane 5 : pJG205 early
Lane 6 : " late
Lane 7 : control (*B.subtilis* commercial prep).

pJG314 shows a range of bands corresponding to β-glucanase activity (lanes 1 and 2). The second smallest band (arrowed), corresponds to the band that was visible on the Western blot. The lower band, at 22KDa, represents fully processed β-glucanase, with the Glu-Ala repeats removed by the action of the STE13 gene product. The two higher bands (approximately 28KDa and 34KDa), are potentially glycosylated forms of β-glucanase. These molecular weights correspond to the addition of 2 and 4 core oligosaccharide units respectively (one core unit is estimated to increase the molecular weight of a protein by approximatly 3KDa (Melnick *at al* 1990)). In lane 2, a fifth band appears, just above 34KDa. This could represent further glycosylation of the enzyme or possibly β-glucanase with the α-factor pro region still intact.

Extracts from the bacterial signal peptide constructs, pJG317 and pJG205, are also shown (lanes 3 to 6). These lanes were overloaded in order to highlight the higher molecular weight bands. In pJG317, a very strong band corresponding to unmodified β-glucanase is visible. Two further bands, at 27KDa and 33KDA approximately, are also apparent. These were not detected on Western blots. Similar to pJG314, these bands correspond to the addition of 2 and 4 core glycosylation units. The disappearance of the larger band late in the cell cycle (lane 4), could possibly be due to its secretion.

In pJG205, no higher molecular weight bands are visible at any concentration of extract, early or late in the growth cycle. This evidence suggests that pJG205 produces no active glycosylated β-glucanase intracellularly. In pJG317, although there is some evidence of glycosylation, a large proportion of the intracellular β-glucanase enzyme remains unmodified. This is in contrast to pJG314 where a more even spread of activity throughout the different forms is observed (Fig.3.25), lanes 1 and 2).

To investigate which forms of the β-glucanase enzyme in each these strains are secreted, supernatant samples were also examined. Figure (3.26) shows the extracts with the corresponding supernatants run alongside. Lanes 2 and 5, show

that glycosylated and unglycosylated β-glucanase is secreted in pJG317 and pJG314 (unglycosylated bands are indicated with arrows). In this gel (15%) (and in the gel of supernatants in Fig.3.27), the slightly slower mobility of the enzyme in pJG314 is more apparent. The analysis also reveals that in each case, the same forms of β-glucanase are secreted as were seen intracellularly.ie. an unglycosylated form and the two core glycosylated forms. In pJG205, the secreted enzyme is similar to the internal form and there is no sign of any modification by oligosaccharide addition under normal growth conditions. This is seen in the supernatant fraction of pJG205 shown in Fig.3.27, (lane 8, minus tunicamycin), where only one band is visible.

Figure (3.26) Activity gel analysis of intracellular and secreted forms of β-glucanase in pJG317, pJG314.

Lane 1: control (*B.subtilis* commercial prep.)
Lane 2: pJG317 intracellular. Lane 4: pJG314 intracellular.
Lane 3: pJG317 extracellular. Lane 5: pJG314 extracellular.

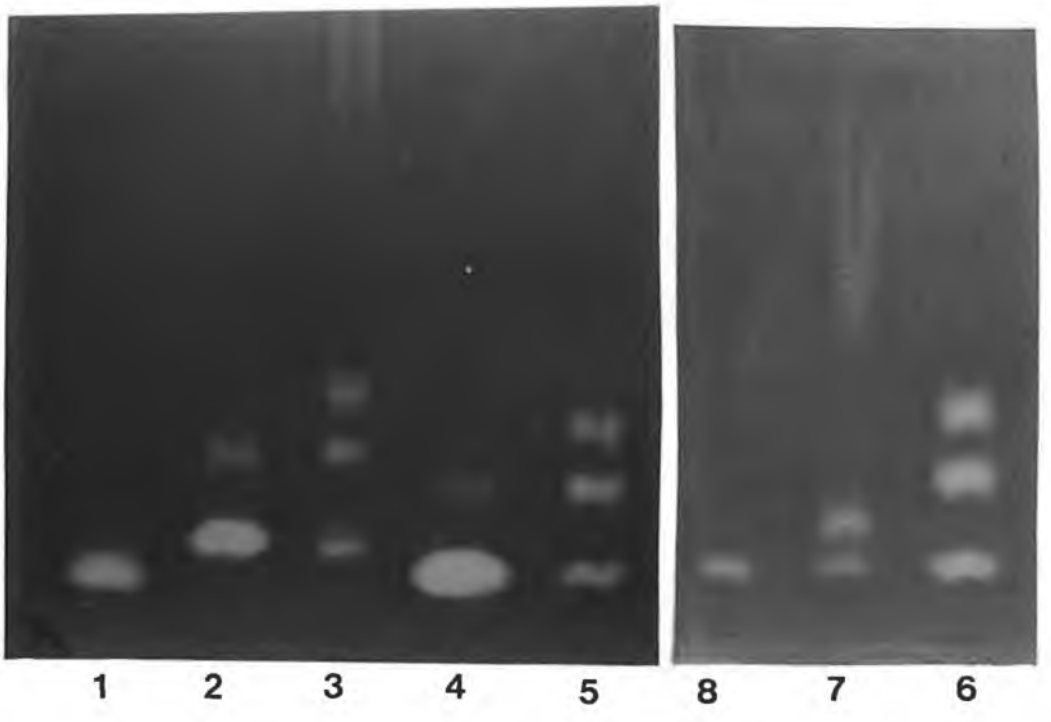
Examination of the β-glucanase products on activity gels after treatment with tunicamycin (Fig.3.27), confirms that the high molecular weight forms seen in the supernatants of pJG317 and pJG314 do represent glycosylated β-glucanase. In the absence of tunicamycin, the majority of the secreted enzyme in pJG317 and pJG314, consists of the higher molecular weight species and in both cases, the higher molecular weight bands are more intense than the unmodified bands (lanes 3 and 5, Fig.3.27). Treatment with tunicamycin causes these bands to decrease in intensity. This is accompanied by an increase in concentration of the low molecular weight, unmodified β-glucanase band. This is apparent in both pJG317 and pJG314 (lanes 2 and 4).

The addition of mannose side chains leading to extensively glycosylated β-glucanase is not evident in either pJG317 or pJG314. Hyperglycosylation would cause a dramatic increase in molecular weight of the enzyme. However, faint smears of activity at molecular weights > 58KDa are seen in pJG317 and pJG314 (lanes 3 and 5; Fig.3.27). These smears may represent extensively glycosylated enzyme and the high level of glycosylation in these bands could possibly have an inhibitory effect on β-glucanase activity. The increases in activity, observed under conditions where glycosylation is disrupted (sections (3.10) and (3.11)), may be a consequence of the release of this fraction of β-glucanase from the inhibitory effects of extensive glycosylation.

In pJG205, tunicamycin treatment results in the appearance of a band of molecular weight 26-27KDa (Fig.3.27, lane 7), slightly smaller than any of the bands detected with the other β-glucanase constructs. Furthermore, this band is not found for pJG205 samples in the absence of tunicamycin and its appearance coincides with the increase in activity detected after tunicamycin treatment (section 3.11). Although no glycosylated forms of β-glucanase for pJG205 were detected using Western analysis or activity gels in the absence of tunicamycin, the *sec*53 data, combined with the tunicamycin assay data for this strain (sections 3.10 and 3.11 respectively), strongly suggest that glycosylation of the enzyme is occurring. This 26-27 KDa

band must therefore represent an early intermediate in glycosylation of β-glucanase in pJG205.

Fig.(3.27). Activity gel of supernatants from pJG317, pJG314 and pJG205 with and without tunicamycin treatment.

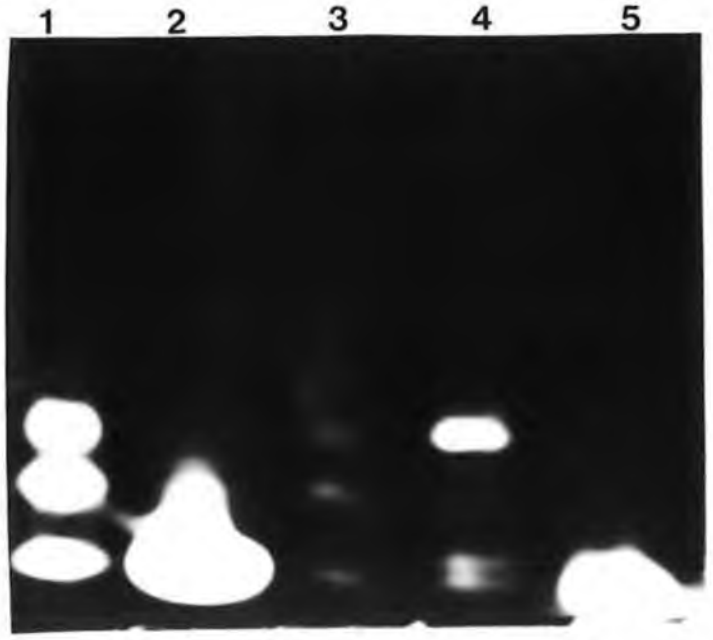


Lane 1: control (*B.subtilis* commercial prep.).
Lane 2: pJG314, + tunicamycin.
Lane 3: pJG314, - tunicamycin.
Lane 4: pJG317, + tunicamycin.
Lane 5: pJG317, - tunicamycin.
Lane 6: pJG317, - tunicamycin (repeat).
Lane 7: pJG205, + tunicamycin.
Lane 8: pJG205, - tunicamycin.

Samples isolated from pVT314 (containing the α-factor signal and ADH1 promoter) were also examined on activity gels to investigate the nature of the β-glucanase enzyme produced by this strain. Figure (3.28) (lanes 1 and 3), show that three active bands are present in the supernatant of pVT314, similar to those observed for pJG317 and pJG314. Intracellularly, two of these bands are visible in pVT314 (lane 4).

The α-factor signal peptide included in this construct, differs from the pJG314 signal in that a fusion was generated placing the β-glucanase gene directly in front of the α-factor Lys-Arg cleavage site (section 3.5). The intervening Glu-Ala repeats are not present in the α-factor pro region in pVT314 and this is reflected in the sizes of the β-glucanase bands observed. Figure (3.28) shows that the smallest unglycosylated β-glucanase in pVT314 is directly comparable in size to the β-glucanase control (lanes 4 and 5) and the three secreted forms of the enzyme are comparable to the three secreted forms in pJG317 (lane 1).

Figure (3.28). Activity gel showing pVT314 intracellular and extracellular fractions.



Lane 1: pJG317 (Sup). Lane 3: pVT314 (Sup).

Lane 2: pJG317 (Ext). Lane 4: pVT314 (Ext).

Lane 5: control (*B.subtilis* commercial prep.).

Section (3.2)

Preliminary studies on the suitability of an *E.coli* β-glucuronidase as a reporter gene for *S.cerevisiae*.

(3.14) **β-glucuronidase as a reporter enzyme for** *S.cerevisiae*.

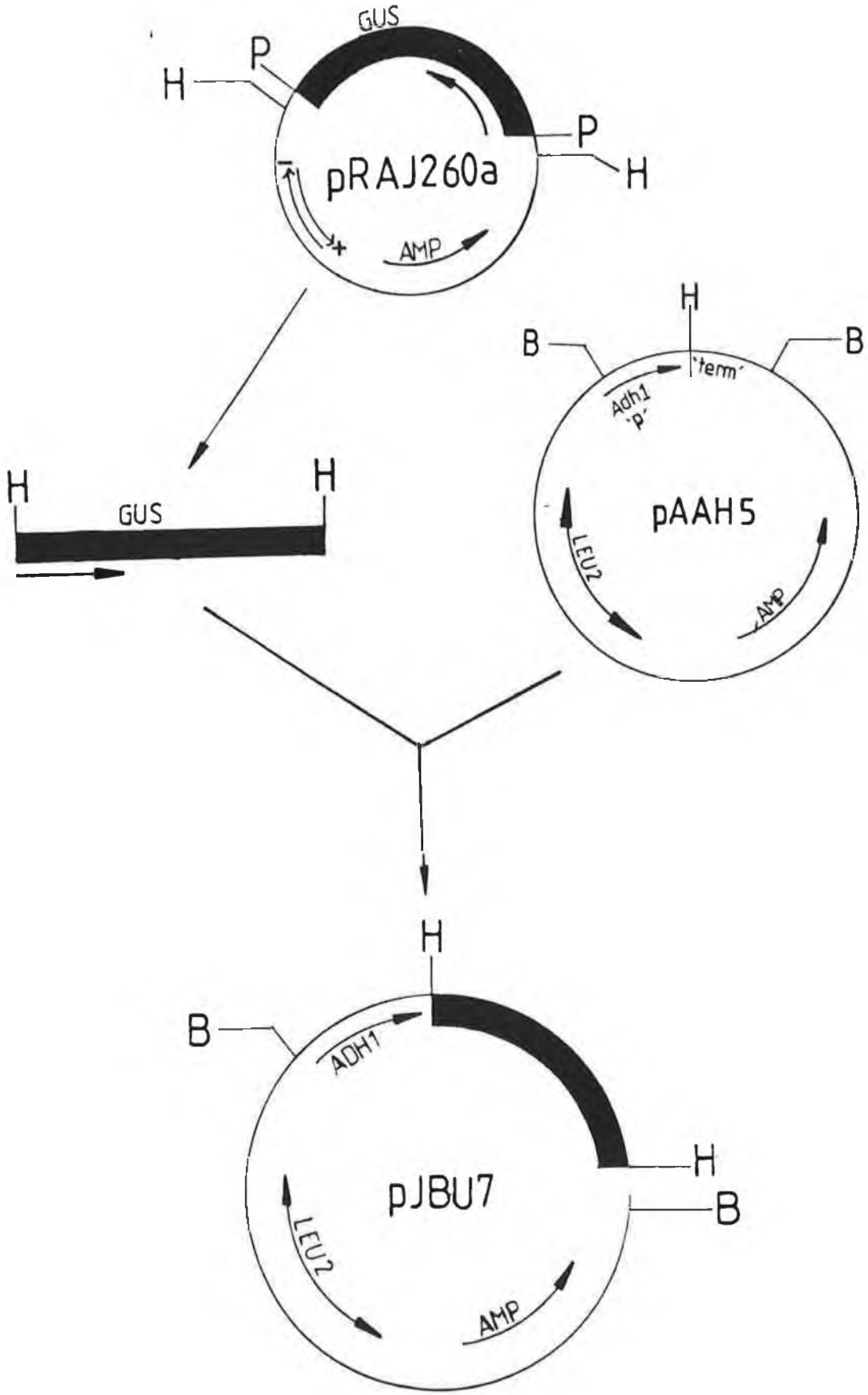
The *E.coli* acid hydrolase, β-D-glucuronidase (EC 3.2.1.31) has been cloned and adapted for use as a reporter enzyme to examine foreign gene expression in plants (Jefferson *et al*. 1986 and 1987). The adaptability of the enzyme to this role and the versatility of the assays available for quick and accurate quantitation of activity, prompted the investigation of its potential as a reporter gene for yeast.

Two plate assays are available for the identification of *E.coli* cells producing β-glucuronidase. The chromogenic substrate, X-gluc (5-bromo-4-chloro-3-indolyl glucuronide), can be incorporated into plates and positive colonies appear blue (section 2.30). Cells sprayed with the flourimetric substrate MUG (4-methyl umbelliferyl glucuronide), flouresce when viewed under UV. This flourimetric assay can also be used to quantify enzyme production. In addition to these plate assays, a simple spectrophotometric assay is available whereby the production of p-nitrophenyl from the substrate p-nitrophenyl glucuronide (PNPG), can be quickly and accurately assessed (section2.30). This simple assay would be ideal for application to the β-glucanase secretion systems described. Investigations were made to assess the potential of introducing β-glucuronidase as a reporter enzyme into *S.cerevisiae*.

(3.15) **Expressing β-glucuronidase in** *S.cerevisiae*.

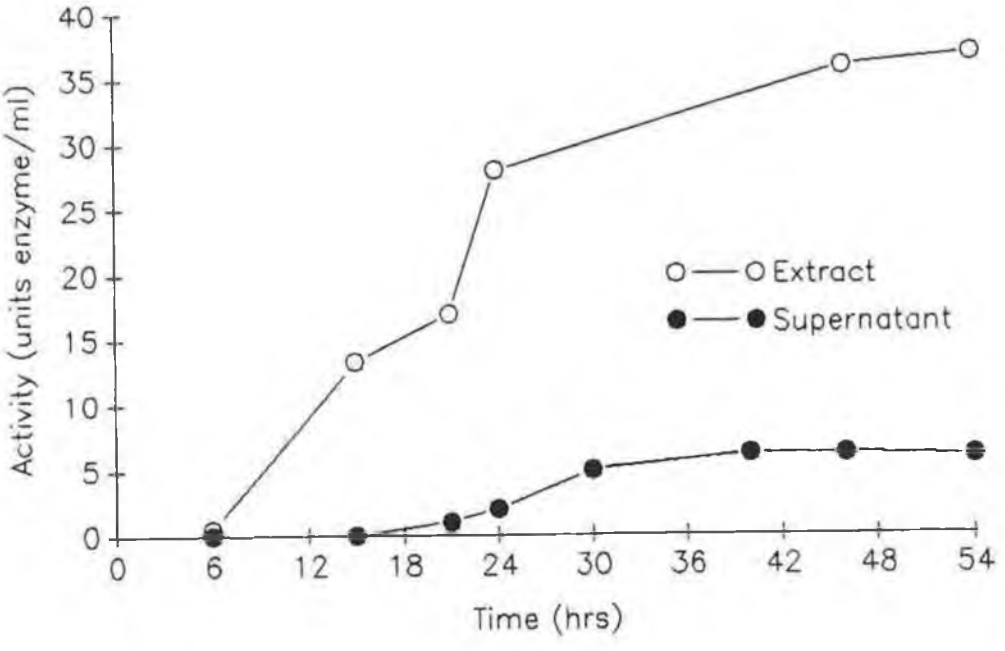
The *E.coli* β-glucuronidase (β-GUS) gene was obtained in pRAJ260 as a 1,800bp *Pst*I fragment in pEMBL9 (Jefferson *et al*. 1986). A *Hin*dIII linker was inserted into the *Sma*I site of pRAJ260 to give pRAJ260a. The β-GUS gene was isolated from pRAJ260a on a *Hin*dIII fragment and ligated into the cloning site of pAAH5 under control of the ADHI promoter (Fig. 3.29). Orientation was confirmed by *Bam*HI digest and pJBU7 transformed into DBY746.

Figure (3.29). Construction of pJBU7.



β-glucuronidase production in pJBU7 was monitored over a 48hr growth period (Fig. 3.30). The spectrophotometric assay (section 2.30), was used to measure enzyme activity. The β-GUS gene appears to be expressed well in yeast and intracellular production levels reach 40units/ml (1 unit is equivalent to 5ng). A low level of extracellular activity was detected during stationary phase, presumably due to a certain amount of cell death and lysis late in the growth cycle.

Figure (3.30). β-glucuronidase production in pJBU7.

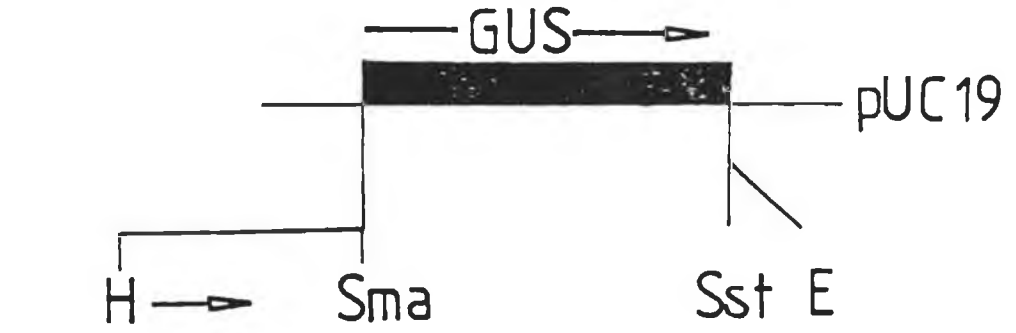


Adaptation of the plate assays to β-glucuronidase production in DBY746 however, proved less successful. pJBU7, when grown in the presence of X-Gluc, does not appear blue in colour. Also, spraying colonies with the flourimetric substrate MUG, does not produce flourescence under UV illumination. It is likely that the yeast cell wall is not permeable to the substrates X-Gluc and MUG. However, pJBU7 in liquid culture does flouresce under UV after incubation in 1mM MUG. This is due presumably to the the low level of extracellular activity that can be detected in the medium, late in the growth cycle. This suggests that the plate assays could be useful, if the β-glucuronidase enzyme was being secreted from the cells. In order to assess the potential of β-glucuronidase to monitor secretion in *S.cerevisiae*, the β-GUS gene was placed under control of the α-factor signal peptide in p69B.

(3.16) **Fusion of β-GUS to the α-factor signal peptide.**

pGUS1, pGUS2 and pGUS3 contain the β-GUS gene inserted into the the multiple cloning site of pUC19 (Fig.3.31). In each case the gene is present in a different reading frame with respect to the *Hin*dIII site. This was achieved by introducing extra nucleotides into the *Sma*I site as indicated (T.Kavannagh, T.C.D).To aid further manipulations and to suit the vectors being used in this study, a *Hin*dII linker was added to the filled in ends of the *Eco*RI site of each plasmid to generate pGUS1a, pGUS2a and pGUS3a.

Figure (3.31) The pGUS series of plasmids.



pGUS1 GGATCCCC G-GGT-GGT-CAG-TCC-CTT-ATG----GUS------

pGUS2 GGATCCCC GG-GTA-GGT-CAG-TCC-CTT-ATG----GUS------

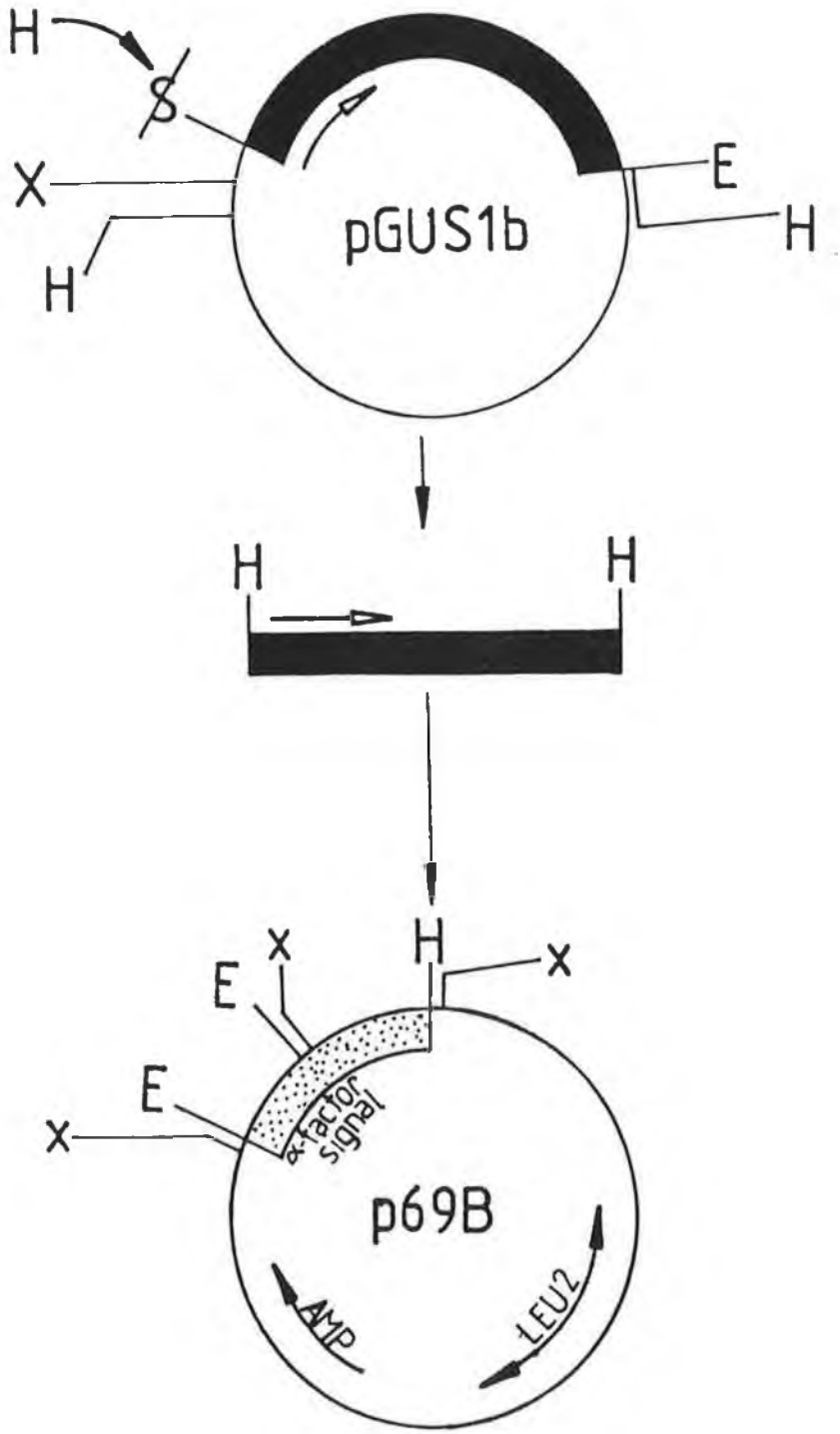
pGUS3 GGATCCCC GGG-TAC-GGT-CAG-TCC-CTT-ATG----GUS------

↑↑

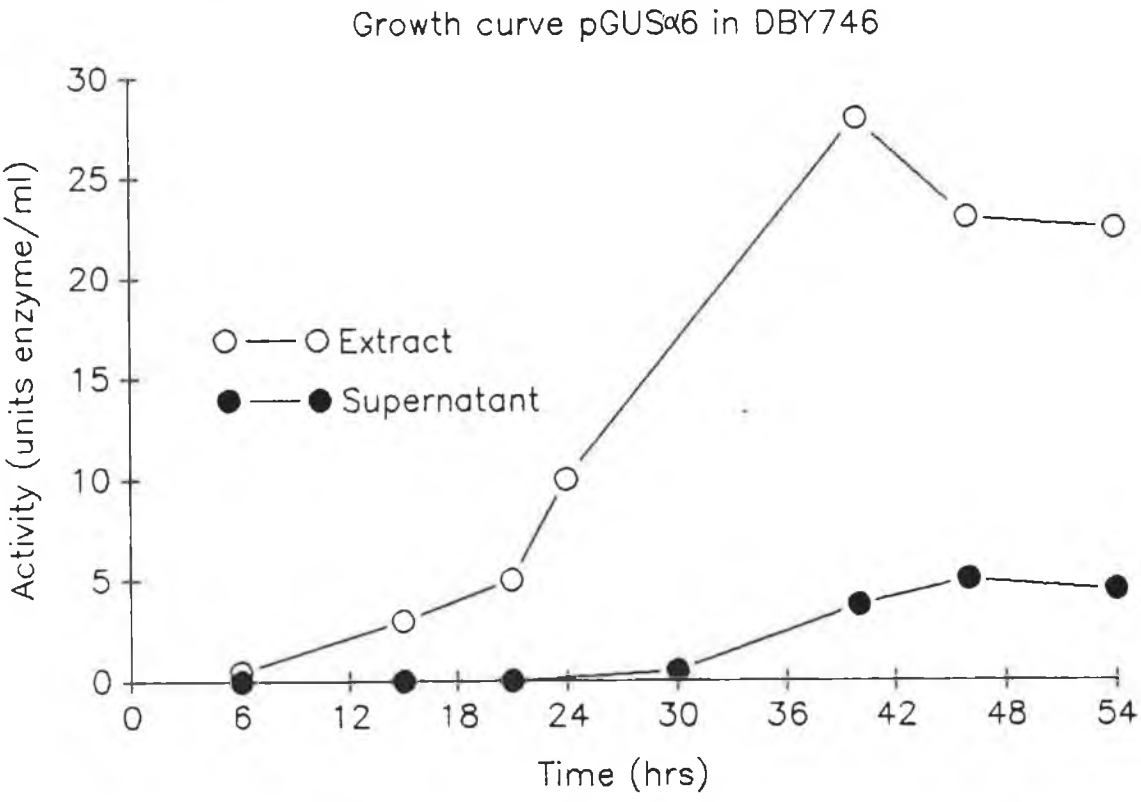
*Bam*HI *Sma*I (extra bases in bold)

To construct the fusion between the α-factor signal peptide and the β-GUS gene, a *Hin*dIII linker was first inserted into the *Sma*I site of pGUS1a to form pGUS1b. The β-GUS gene was then isolated on this smaller *Hin*dIII fragment and ligated into p69B to give pGUSα6 (Fig.3.32).

Figure (3.32) Construction of pGUSα6.



Analysis of β-glucuronidase production in pGUSα6 was monitored over a 48hr growth period (Fig.3.33). Levels of intracellular activity are lower overall than pJBU7 and no extracellular activity, apart from the very low levels comparable to those detected in the signal-less construct pJBU7, was observed. Figure (3.33).



To ensure that glycosylation was not having a similar effect on β-glucuronidase as had been seen with β-glucanase (the β-glucuronidase enzyme has two potential glycosylation sites), pGUSα6 was treated with tunicamycin (10μg/ml) as described section (3.11). Tunicamycin had no effect on β-glucuronidase activity produced from pGUSα6 intracellularly or extracellularly. It appears that the β-glucuronidase enzyme cannot be secreted in *S.cerevisiae* using the α-factor signal peptide. However, these results do give some idea about the relative strengths of the ADH1 and α-factor promoters. If the α-factor signal peptide does not interfere with β-glucuronidase activity in pGUSα6, then the ADH1 promoter is the stronger of the two.

(3.17) **Adapting β-glucuronidase as a useful reporter in yeast.**

β-glucuronidase does not appear to be amenable to monitoring secretion in *S.cerevisiae*. In the yeast cytoplasm however, the enzyme shows no signs of instability and can be used to monitor expression levels. β-glucuronidase may also be useful in other respects for heterologous protein production in *S.cerevisiae*. For example, the enzyme could possibly be used as a fusion protein to act as a carrier for other foreign proteins in yeast. It may also be possible to stabilize proteins, eg.β-glucanase, by fusion to the β-glucuronidase enzyme.

β-glucuronidase is a 73KDa protein, and a smaller protein would be potentially more useful for fusion studies. If a truncated, but still active form of the enzyme could be generated, this would be useful both as a fusion to stabilize other proteins in the yeast cytoplasm and to investigate the yeast secetory pathway further. If the limiting factor for secreting β-glucuronidase in yeast is its size, truncated forms of the enzyme would be particularly useful. Alternatively, if there are sequences in the enzyme that direct the protein elsewhere in the yeast cell, truncated forms would also aid the investigation of this possibility.

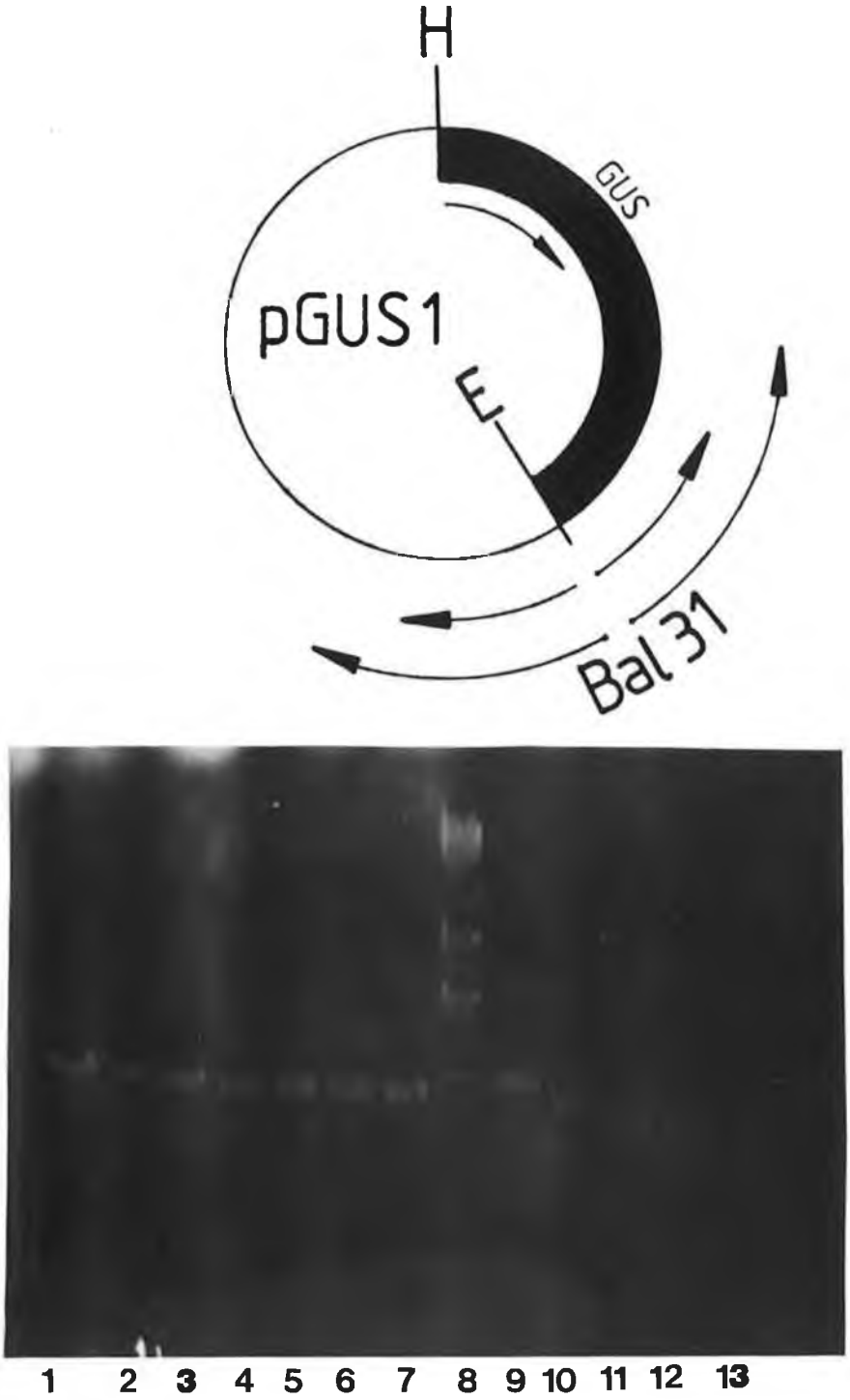
An earlier observation involving β-glucuronidase suggested that it should be possible to produce a shortened form of the enzyme that was still active (T.Kavanagh, T.C.D.). On activity gels, active enzyme bands were observed below the main active band (72KDa) in partly degraded preparations of the enzyme. This indicated that the active domain of the enzyme was confined to a shorter defined region of the molecule. Initial steps were taken to locate the active site of β-glucuronidase using *Bal*31 deletion analysis from both the N-terminal and carboxy terminal of the enzyme. Antibody to the *E.coli* enzyme was also raised to aid future investigations in this area and a fusion protein involving the β-glucanase and β-glucuronidase genes was constructed.

(3.18) **Truncating β-GUS from the 3' end.**

pGUS1 was linearised with *Eco*RI and deletions were made into the 3' end of the gene using *Bal*31 exonuclease as described in section (2.15) and Figure (3.34). Digestion was stopped at times T=2 to T=20 mins (Fig.3.34) and the samples cleaned by spinning through sepharose columns. The linear plasmids from T=10 were relcircularised in the presence of a *Hin*dIII linker and transformed into JA221. Transformants were tested for β-glucuronidase activity using the flourescent MUG assay (section 2.30). Deletions from the 3' end disrupted activity of the enzyme almost immediatly. Even the smallest deletion of 150bp was inactive in *E.coli*. Two of these truncated genes were isolated on *Hin*dIII fragments and ligated into the *Hin*dIII site of pAAH5 between the ADH1 promoter and terminator sequences. These deletions pJBUΔb and pJBUΔi, missing 150bp and 500bp of the β-GUS gene respectively, not surprisingly, exhibited no activity in DBY746 (not shown).

The carboxyl end of the β-glucuronidase appears to be essential for activity.This suggests that the active site of the enzyme is located in this region. The β-GUS gene has two potential glycosylation sites also located in this area, at positions 1,200 and 1,400 bp approximatly (Jefferson *et al*. 1986). Glycosylation of β-glucuronidase at these two sites in the ER of plant cells has been shown to have an adverse effect on activity and is consistent with this observation (Ittaguria *et al*, 1990). These studies carried out in plants, also show that the enzyme is not adverse to translocation, at least in this higher eukaryote.It may be possible therefore, to secrete β-glucuronidase in yeast, perhaps using a different signal peptide.

Figure (3.34) 3′ *Bal*31 deletions of β-GUS gene.



Lane 1 : pGUS1 / *Hin*dIII.

Lanes 2-7 : *Bal*31 deletions, T= 2, 4, 6, 8, 10, 12 mins.

Lane 8: λ / *Hin*dIII

Lanes 9-13 : *Bal*31 deletions, T= 14, 16, 18, 20 mins.

(3.19) **Truncating β-GUS from the 5' end.**

To generate 5' deletions in the β-GUS gene, pGUS2, cut with *Hin*dIII, was treated with *Bal*31 exonuclease (section 2.15) (Fig.3.35). Digestion was stopped at times T=2-20 mins. and the samples cleaned by spinning through sepharose columns. Because the translational start site is automatically removed in this procedure, the deleted plasmid (from T=10) was recircularised in the presence of a *Cla*I linker to generate an ATG start site and transformed into JA221.

Figure (3.35). Generation of 5' *Bal*31 deletions.

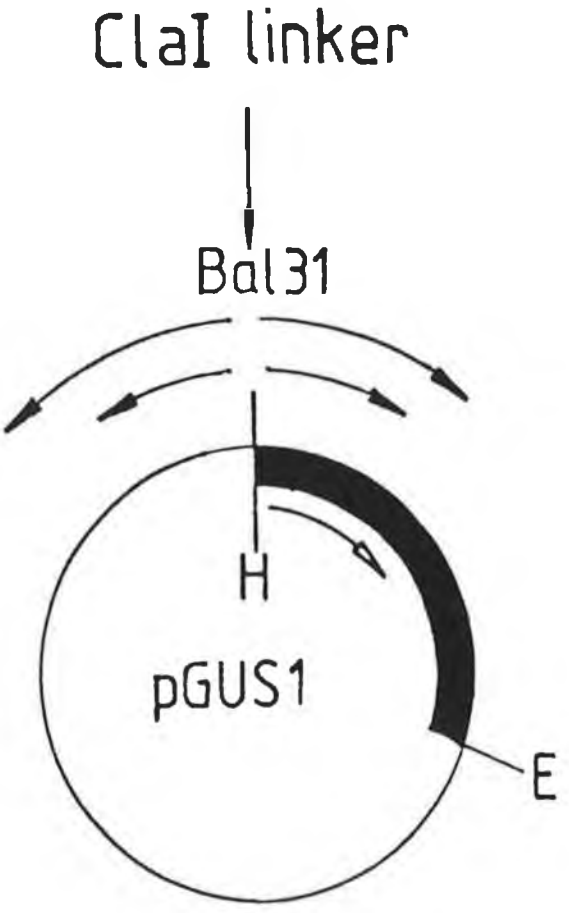
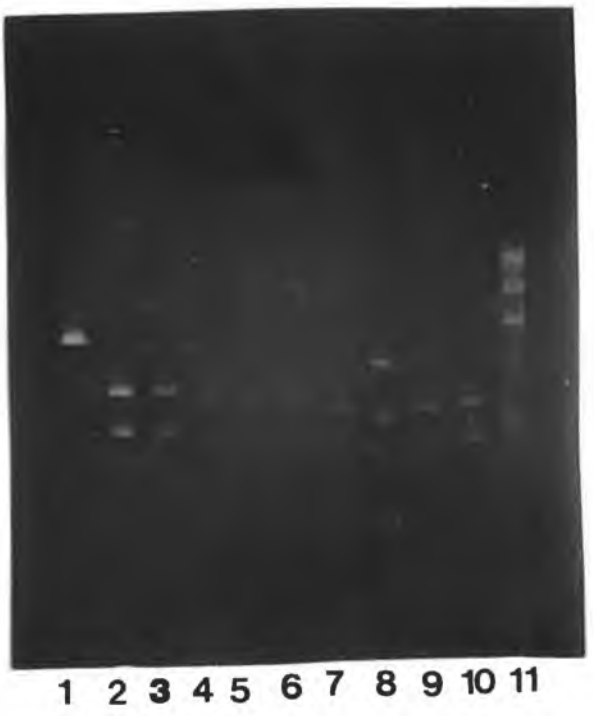


Figure (3.35) contd.: Gel showing *Bal*31 deletions T=2 to T=20 mins., after digestion with *Eco*RI to separate vector and β-GUS gene. T=10 was taken for religation in the presence of *Cla*I linker.



Lane 1: pGUS1 / *Hin*dIII (linearized).

Lane 2: pGUS1 / *Hin*dIII / *Eco*RI.

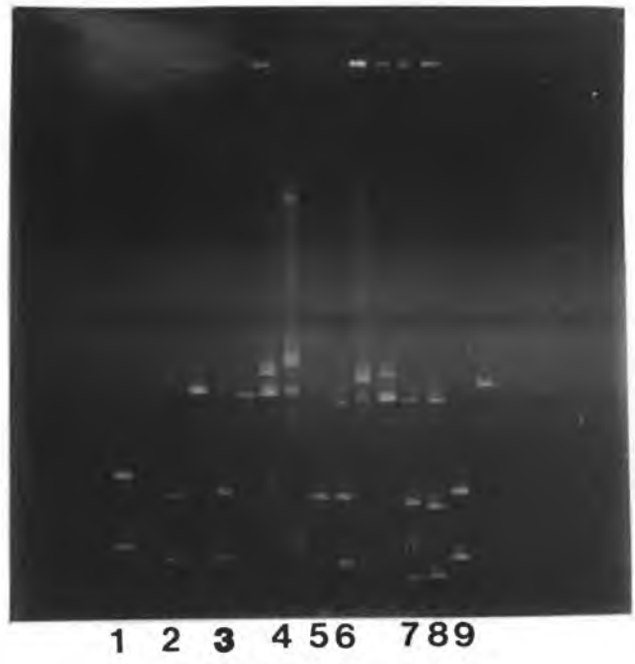
Lanes 3-9: *Bal*31 deletions, T= 2, 4, 6, 8, 10, 20, 15 mins.

Lane 10: pGUS1 / *Hin*dIII / *Eco*RI.

Lane 11: λ / *Hin*dIII.

Analysis of the religated T=10 transformants show that between 200 and 600bp of the gene has been removed by the action of the *Bal*31 enzyme. Figure (3.36) shows 8 of these transformants cut with *Cla*I and *Eco*RI.

Figure (3.36)

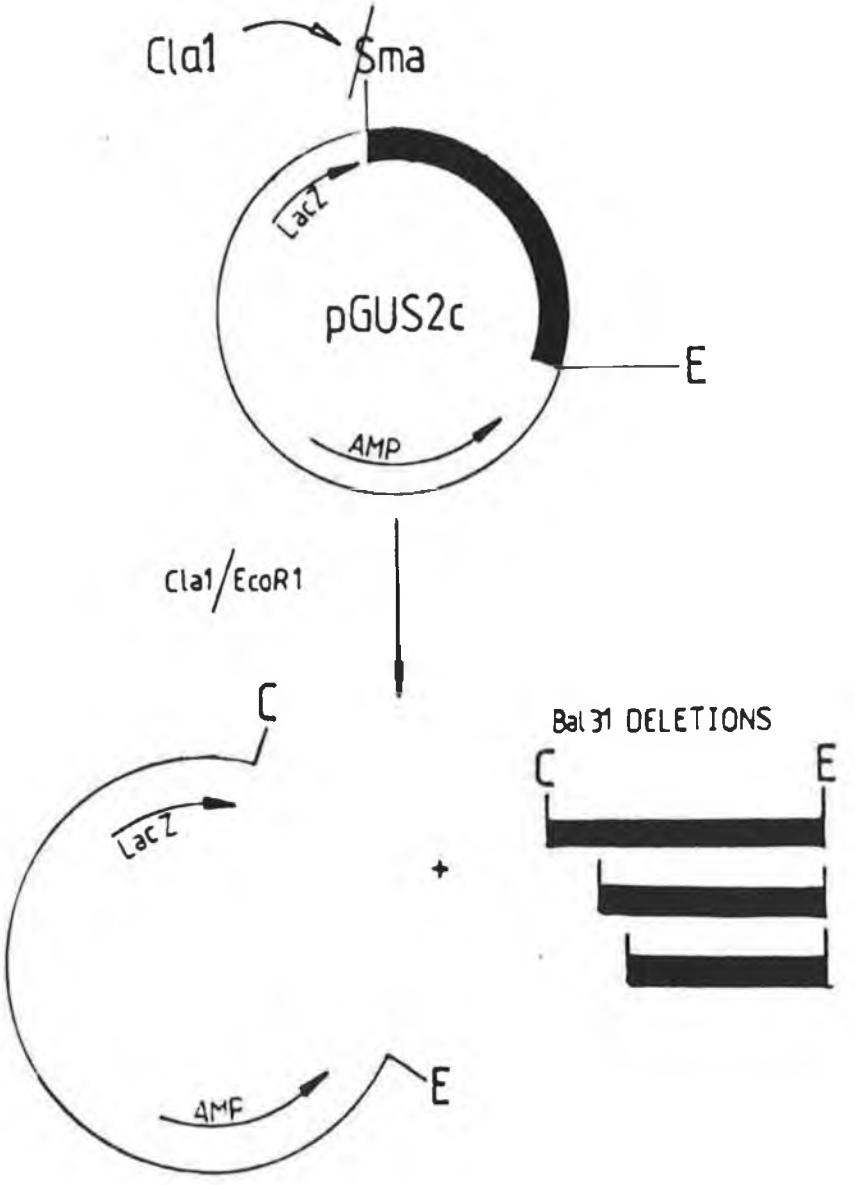


Lane 1: pGUS1 *Cla*1 / *Eco*RI.
Lane 2: pΔ16.13
Lane 3: pΔ16.15
Lane 4: λ / *Hin*dIII / *Eco*RI.
Lane 5: pΔ16.1
Lane 6: pΔ16.2
Lane 7: pΔ16 6
Lane 8 pΔ16.9
Lane 9: pΔ16.10

All the above deletion fragments were gel purified and cloned into the pGUS2c vector as described below.

These truncated genes (from Fig.3.36) were isolated on *Cla*I/*Eco*RI fragments and ligated into the vector portion of pGUS2c. pGUS2c was constructed to mediate transfer of the truncated β-GUS genes into pUC19 under control of a fully intact lacZ promoter (Fig.3.37): A *Cla*I linker was inserted into the *Sma*I site of pGUS2 and the vector portion of the resultant plasmid (pGUS2c), was gel purified after digestion with *Cla*I and *Eco*RI. The truncated β-GUS genes were ligated to this vector and transformed into JA221 (JA221 has no endogenous β-glucuronidase activity).

Figure (3.37). Construction of pGUS2c.



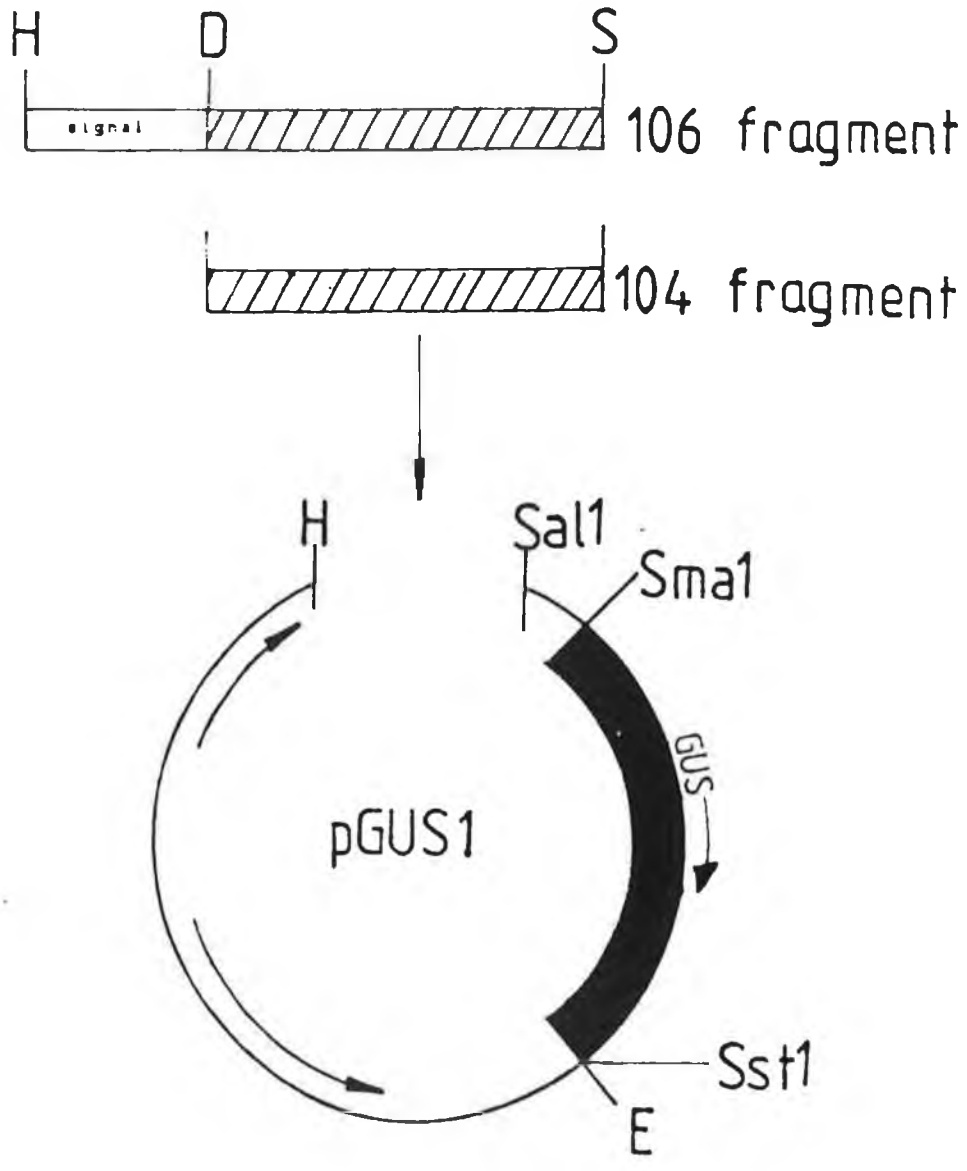
When tested for β-glucuronidase acitvity using the flourescent MUG assay (Fig.3.38), two of the deletions were shown to have retained activity ie. pΔ16.1 and pΔ16.9, missing approximately 500bp and 300bp of the β-GUS gene respectively (see Fig.3.36). This indicates that at least 500bp of the β-GUS gene can be removed without the enzyme losing its activity and it would obviously be worthwhile to continue the deletion analysis further into the 5′ end of the gene. The lack of activity in the other deletions, where less than 500bp has been removed, is likely to be a result of out of frame fusions between the deleted gene and the translational start site of the *Cla*I linker.

Figure (3.38). MUG assay of *Bal*31 deletions.
To test for β-glucanase activity, the cultures were mixed with 1mM MUG in microtitre wells, incubated at 37^{0}C for 20 mins and viewed under UV.

(3.20). **β-glucuronidase as a fusion protein.**

To investigate if β-glucuronidase can tolerate a 5′ fusion, the β-glucanase gene was ligated in-frame to the 5′ end of the β-GUS gene in the plasmid pGUS1. In generating this fusion, it was necessary to ligate the two genes in-frame, eliminating all transcriptional and translational termination sequences between the two. This was achived by isolating the β-glucanase gene on a *Hin*dIII-*Sal*I fragment from both pJG104 and pJG106, as outlined in Figure (3.39). Both plasmids were used in order to generate fusions between β-glucuronidase and the β-glucanase gene, with and without its signal peptide. Truncating the β-glucanase gene at the *Sal*I site (position 1115, app.1 for details) results in the removal of 54 amino acids of the β-glucanase carboxyl terminus. Whether or not this destroyed the active site of the β-glucanase enzyme remained to be determined. However, the complete β-glucuronidase gene would be present in these fusions and it was hoped that this enzyme would remain active.The truncated β-glucanase genes were each ligated into *Hin*dIII-*Sal*I cut pGUS1, 5′ to the β-GUS gene, generating an in-frame fusion between the two enzymes in each case (Fig.3.39). The resultant plasmids pJGUS4.1 (containing the signal-less β-glucanase) and pJGUS6.1 (with the β-glucanase signal included), were transformed into JA221, replica plated and tested for β-glucanase activity using the plate assay (section 2.18) and for β-glucuronidase using the flourescent MUG assay (section 2.30). In both cases, the fusions were positive for β-glucuronidase and negative for β-glucanase activity. This indicates that β-glucuronidase can be made as an active fusion to other peptides. These fusions have yet to be tested in yeast.

Figure (3.39). Construction of β-glucanase / β-glucuronidase fusion plasmids.



(3.21) **Raising a polyclonal antibody to the *E.coli* β-glucuronidase enzyme.**

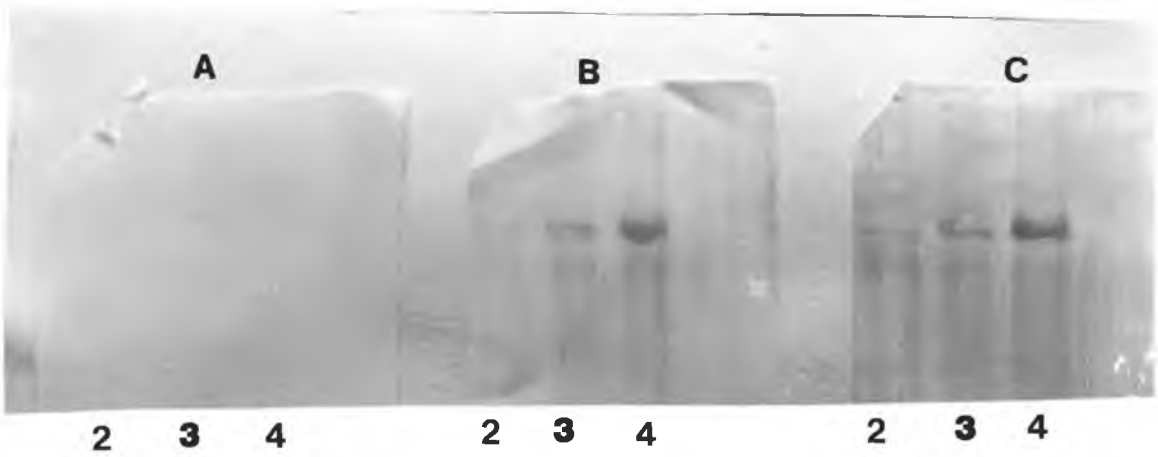
An activity gel technique is available for analysis of the β-glucuronidase protein using SDS PAGE (section 2.31). This technique is based on the flourescent MUG assay where flourescing bands of renatured β-glucuronidase are viewed by UV illumination. This technique however, is unreliable and the speed at which the flourescence fades under UV hinders it even further. In order to facilitate the future analysis of the full, truncated and fusion forms of β-glucuronidase, it was decided to make a polyclonal antibody to the enzyme for analysis in *E.coli* and yeast.

A commercial preparation (Sigma) of the *E.coli* β-glucuronidase enzyme was analysed by SDS PAGE. Coomassie blue staining showed this preparation to be largely free of contaminating proteins (data not shown). A 72 day old rat was injected sub-cutaneously with 500 units (in 0.5ml H_2O) of this enzyme preparation, sonicated in 0.5ml complete Freunds adjuvunct. A booster injection of the same amount, this time sonicated in 0.5ml Freunds incomplete adjuvunct, was subsequently administered at 3 weekly intervals, 5 times in total. One week after the last injection, 1.0ml of serum was removed, allowed to clot overnight at 4^0C and cleared by centrifugation. The cleared antiserum was tested for reactivity against the commercial β-glucuronidase enzyme using Western blot analysis. On establishing reactivity of the antiserum with the β-glucuronidase protein, 15ml of blood was extracted from the rat. This was allowed to clot and the cleared serum collected after centrifugation as before. A total of 8.0ml of antiserum was recovered.

The antibody preparation was titred against various concentrations of commercial β-glucuronidase by Western blot analysis. 30, 60 and 180 units of the enzyme preparation were separated on a 15% SDS polyacrylamide gel, blotted onto nitrocellulose and developed using a 1:200, 1:500 and 1:1,000 dilution of β-glucuronidase antiserum. As a control, non-immune rat serum was also used to rule out non-specific binding of the

antibody. Figure (3.40) shows the blot of the 1:1,000 dilution which gave the best results with respect to intensity of bands and clearness of background. Hybridization to the non-immune rat serum control showed that non-specific binding was at a minimum.

Figure (3.40). Titring the β-glucuronidase antibody (1:1000).

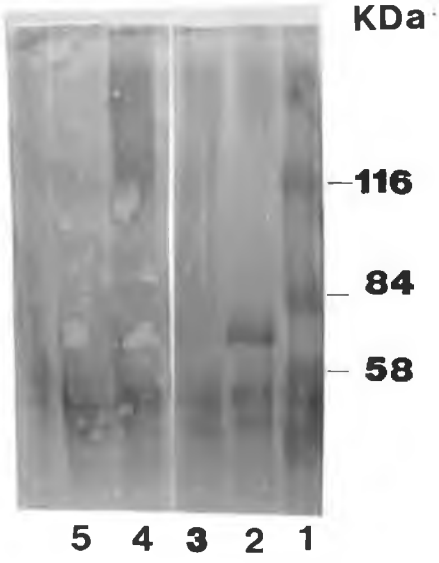


A = negative control, non-immune rat serum.
B = 1:1000 dilution of β-glucuronidase antiserum.
C = 1:500 dilution of β-glucuronidase antiserum.

Lane 1: markers
Lane 2: 30 units *E.coli* commercial β-glucuronidase
Lane 3: 60 units " " "
Lane 4: 180 units " " "

To ensure that the antibody also recognises β-glucuronidase produced in the yeast cytoplasm and to compare the sizes of the *E.coli* and *S.cerevisiae* enzyme, the antibody was tested against a protein extract prepared from pJBU7. The Western blot, shown in Figure (3.41) reveals a band at the same mobility in *E.coli* and pJBU7 (72KDa). pAAH5 was also run to ensure against non-specific binding and the same blot was tested against non-immune rat serum. The faintness of the band in pJBU7 is likely to be a result of the low amount of extract run on the gel (approximately equivalent to 200μl of a stationary phase culture). This shows however, that the antibody does recognize the enzyme when produced in yeast, even at low concentrations and should prove useful in future investigations of β-glucuronidase production *S.cerevisiae*. Contaminating bands that appear in the commercial preparation of the enzyme, running at a faster mobility than β-glucuronidase, also appear in the yeast extract samples. These bands are most likely to be a result of non-specific binding of the antibody preparation and are not associated with β-glucuronidase activity.

Figure (3.41). Reactivity of the β-glucuronidase antibody with enzyme produced in yeast (1:1,000 dilution of antibody).



Lane 1: markers

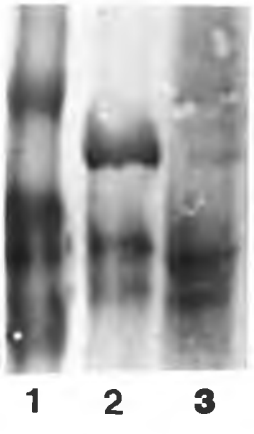
Lane 2: *E.coli* commercial prep.

Lane 3: pJBU7 extract.

Lane 4: DBY746 extract.

Lane 5: pAAH5 extract.

Close up of lanes 1, 2 and 3 showing the β-glucuronidase band in pJBU7 more distinctly.

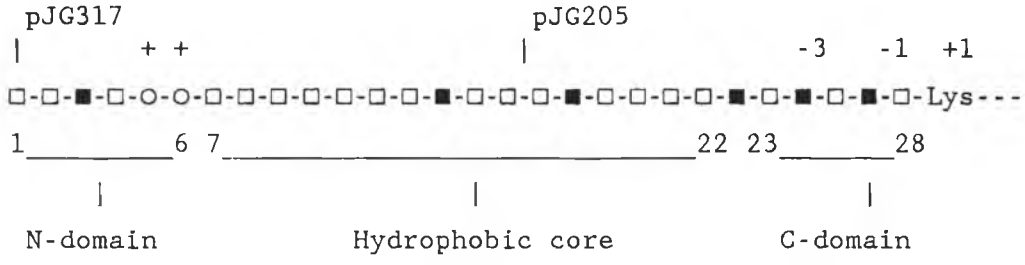


CHAPTER 4
Discussion

(4.1) The β-glucanase signal peptide.

The *B.subtlis* signal peptide has been shown to act as a natural signal sequence in *S.cerevisiae*, directing secretion of β-glucanase through the conventional secretory pathway. This was verified with pJG317, in *sec*18 and *sec*1 mutants, where secretion of the β-glucanase enzyme was blocked at the initial and final stages of the transport system. The bacterial signal peptide is typical of prokaryotic and eukaryotic secretory proteins and a physical dissection of the sequence into its structural domains is shown in Figure (4.1).

Figure (4.1). Physical dissection of the β-glucanase signal peptide.



□ = hydrophobic residue; ■ = polar residue, O = basic residue

The N-terminal hydrophillic domain consists of 6 amino acids, ending with two positively charged residues, Lys and Arg. This is followed by a hydrophobic core sequence of 16 amino acids. The C-terminal domain of 6 amino acids contains the signal peptidase cleavage site. Cleavage by signal peptidase is likely to occur after the Ala residue at position 28. Von Heijne (1984b), suggests that the cleavage window begins at 4-5 residues from the end of the hydrophobic core and takes place at the strongest site allowed. According to these rules, there is a strong preference for Ala at positions -1 and -3 and a charged or polar amino acid is preferred at position +1, serine however, is seldom seen at this position. Small uncharged residues predominate at position -2. The sequence Ala-Ser-Ala-Lys at position -3 to +1 fits in exactly with these predictions. Western blot analysis indicated that the bacterial

signal peptide is processed by signal peptidase during translocation of β-glucanase across the ER membrane This is demonstrated in the appearance of a single, processed band in the supernatant of pJG317 after tunicamycin treatment (Fig. 3.24). Although protein sequence analysis would be required to establish the exact cleavage site, according to the general rules, cleavage is most likely to occur at this site.

(4.2) Deletion of the β-glucanase signal peptide.

Complete removal of the β-glucanase signal peptide in JBβ4 and pJBC55A, resulted in a loss of detectable enzyme activity when expressed on a multicopy plasmid in the yeast cytoplasm. In the protease deficient mutant MD50 however, partial recovery of low levels of activity was achieved with pJBβ4.

Recovery of low levels of β-glucanase activity in the cytoplasm in the absence of these proteases demonstrates that without its signal peptide, the β-glucanase enzyme is unstable and is a substrate for proteolysis. *In vivo*, this form of the enzyme may also be a substrate for cellular proteases in the yeast cytoplasm.

Degradation in the cytoplasm is a common problem encountered with the production of foreign proteins in yeast and in higher eukaryotic cells (Townsend *et al* 1986). This has been shown for the yeast secretory protein acid phospatase, where up to 80% of the protein is subject to degradation with the signal peptide removed (Silve *et al* 1987). Removal of the signal peptide in β-glucanase is likely to result in conformational changes in the β-glucanase enzyme, possibly rendering it more sensitive to the action of cellular proteases. The identity of the N-terminal amino acid residue has also been shown to affect protein stability in yeast (section 1.1.2). In pJBβ4 and PJBC55A however, the respective N-terminal Met-Ser-Lys and Met-Gly-Asp sequences, fall into the stabilizing category. Even if the N-terminal Met was removed *in vivo*, the second residue in each case is also stabilizing.

In pJBβ4, a contributory factor to the lack of detectable activity in DBY746 and the low levels of activity produced in MD50, is likely to be the instability of the β-glucanase transcript. This instability is reflected in Northern blots where no β-glucanase specific transcript is detected for pJBβ4. Efficient termination has been shown to be important for RNA stability in yeast and expression levels can increase dramatically when this is achieved (Ammerer, 1983; Zaret and Sherman, 1982). In pJBβ4, the bacterial terminator alone may not terminate transcription efficiently.

pJBC55A on the other hand, produces a remarkably strong β-glucanase transcript, but reveals no sign of any enzyme activity when expressed in DBY746, or in MD50. Examination of the 5' untranslated leader regions of the signal-less gene in pJB55A, suggests that the absence of active enzyme may be associated with translation efficiency. Highly expressed yeast genes tend to contain untranslated 5' leader regions that are short (approximatly 52 nucleotides) and void of secondary structure (Cigan and Donaghue, 1987). A lack of secondary structure is promoted by a bias toward A and a strict avoidance of G nucleotides in this region. These features promote conditions ideal for attachment and movement of the ribosome towards the initiator codon. In addition, a consensus sequence has been identified around the AUG start that has been shown to promote efficient translation, presumably optimizing conditions for recognition of the initiator. This sequence, compiled by examining nucleotide bias around the AUG of highly expressed genes, is also rich in A and void of G nucleotides and takes the form A/Y-A-A/U-A-AUG, where Y is a pyrimidine (U or C). Optimization of the 5' leaders of foreign genes in accordance with these guidelines, has been shown to have dramatic effects, resulting in increased expression levels in yeast (Bitter and Egan, 1988; Fieschko *et al* 1987).

Comparison of the 5' leaders of pJBβ4 and pJBC55A, reveals differences both in their lengths and composition. Assuming that transcription is initiated at the same site as that defined for the ADH1 promoter (between positions -37 and -27,

(Ammerer, 1983)), the 5′ untranslated regions of the β-glucanase transcripts in pJBβ4 and pJBC55A are as shown in Fig.(4.2). The sequences from position -12 are shown to highlight these differences. pJBβ4, is completely void of Gs and exhibits an almost ideal consensus around the AUG start. pJBC55A on the other hand, is longer (by 9 nucleotides), contains 3 G nucleotides and has no consensus at the initiator AUG. These features of pJBC55A may contribute to inefficient translational initiation in pJBC55A compared to pJBβ4. A combination of inefficient translation with a certain amount of protein instability and degradation are possible reasons for the lack of enzyme activity in pJBC55A.

Figure (4.2) 5′ untranslated regions of pJBβ4 and pJBC55A.

pJBβ4

CAAUCAACUAUCUCAUAUACA-AUG-UCU-CAA-AGC-UUG-GGG-GAU-CCG-UCA

from pAH9 Met-Ser-Lys-.....-Ser

pJBC55A

CAAUCAACUCCAAGCUUGAAUUCCCCAUCG-AUG-GGG-GAU-CCU-UCA--

pAAH5 Met-Gly-Asp-----Ser

Sequence around AUG initiator in pJBβ4 and pJBC55A:

pJBβ4	U-A-C-A-AUG
Consensus	A/Y-A-A/U-A-AUG
pJBC55A	A-U-C-G-AUG

(4.3) **Truncating the β-glucanase signal peptide.**

pJG205 contains a truncated version of the β-glucanase signal peptide, starting with the Met residue at position 17 (see Fig. 4.1). This signal peptide, although missing the complete hydrophillic and the bulk of the hydrophobic domains, directs secretion of β-glucanase into the yeast cell medium. *Sec* mutant analysis confirms that the extracellular β-glucanase activity detected in pJG205, is due to transport through the conventional secretory pathway. This suggests that the severely truncated bacterial signal sequence can direct translocation and secretion of heterologous proteins in yeast.

Kaiser *et al* (1987), has proposed that translocation of proteins into the ER only requires the presence of a short stretch of hydrophobic amino acids (section 1.3.2). pJG205 fulfills this obligation with a hydrophobic core of 4 residues. However, Kaisers experiments also showed that the levels of translocation achieved with limited hydrophobicity are extremely low. Initial observations with pJG205, comparing the levels of secreted enzyme to pJG317, suggested that the truncated version of the signal peptide secretes β-glucanase in amounts comparable to the complete sequence, with extracellular levels of enzyme activity being almost the same in each case. This comparison however, is complicated by the very high levels of β-glucanase activity detected intracellularly in pJG205. Taking into account the total amount of β-glucanase activity produced by the cells, the secretion efficiency in pJG205 is only 14%, whereas in pJG317, approximately 50% of the overall levels are secreted. The total amount of activity produced in pJG317 however, is significantly lower than in pJG205 (80-90% lower) (section 3.4).

Examination of the steady state levels of the β-glucanase RNA transcripts by Northern analysis, revealed no significant differences between pJG317 and pJG205. This indicates that the lower levels of activity observed in pJG317 are not due to RNA instability. Also, differences in translation efficiencies are not likely to be the cause of the discrepancies in overall levels of enzyme produced. The two plasmid constructions are

identical except for a region of 10 and 11 base pairs, derived from the β-glucanase gene sequence preceding the initiator methionines. These 5′ untranslated regions, while they do not constitute ideal 5′ leader sequences according to the accepted guidelines outlined in section (4.2) above, are similar in length and contain equal numbers of G nucleotides. Of the two, the pJG317 sequence is more conducive to efficient initiation of translation than pJG205. (The pJG205 sequences 5′ to the initiator Met are: TGGATTGTTTATG ; the pJG317 sequences 5′ to the initiator Met are: GGAATGCCAATATG).

Glycosylation of the β-gluucanase enzyme, in the secretory pathway of DBY746, appears to inhibit activity of the enzyme (see section 4.5). Activity gel analysis suggests that glycosylation of the enzyme produced in pJG205 does not occur to the extent seen in pJG317 or pJG314. Reduced glycosylation may therefore contribute to the high levels of activity observed intracellularly in pJG205.

This lack of glycosylation in pJG205 could reflect inefficient translocation of the β-glucanase enzyme into the secretory pathway, caused by the presence of the truncated signal peptide. Western analysis of pJG205 extracts suggests that there is a substantial amount of unprocessed enzyme, with the signal peptide still intact, present intracellularly in this strain. The wide smear of activity observed on activity gels is also consistent with the presence of processed and unprocessed bands (Fig. 3.25). The fraction that is secreted in pJG205 however, is processed with respect to signal cleavage. This is evident by comparing the size of the extracellular fraction to the *B.subtilis* control enzyme (Fig. 3.26).

(4.4) Comparison of the β-glucanase and α-factor signal peptides in pJG317, pJG314 and pVT314.

Comparison of the full bacterial signal peptide with the yeast α-factor signal, in relation to their abilities to secrete β-glucanase, is also complicated by the problem of glycosylation. The growth curves presented in section (3.4), suggest that the *Bacillus* signal is at least as efficient as the α-factor signal. In pJG317 and pJG314, approximately half of the overall levels produced in each case is secreted. In pJG314, the total amount of β-glucanase produced is approximately 50% of that produced in pJG317. These lower overall levels were assumed to reflect the differences in the relative strengths of the Adh1 and α-factor promoters. However, pJG317 demonstrates that DBY746 is capable of secreting almost 450 units of β-glucanase activity per ml of culture under the conditions used. The lower levels secreted in pJG314 may simply reflect reduced efficiency of the α-factor signal peptide to secrete and process the enzyme.

In an attempt to clarify this and to obtain a more direct comparison of the secretion efficiencies of the bacterial and yeast signal peptides, pVT314 was made. This construct places the β-glucanase gene, fused to the α-factor signal sequence, under control of the Adh1 promoter and was intended to act as a direct control for comparison to pJG317. It was expected that the same promoter in pVT314 and pJG317 would result in similar overall levels of β-glucanase production with the levels secreted reflecting the relative strengths of the two signal peptides. The levels detected in pVT314 however, intracellularly and extracellularly, are significantly reduced. Surprisingly, these overall levels are also lower than those observed in pJG314. Over much of the growth curve the secreted enzyme levels of pVT314 are also lower than those observed in pJG314 despite the fact that in both constructs the β-glucanase gene is fused to the α-factor signal peptide.

Other studies have shown that overexpression of heterologous genes, in combination with certain signal peptides, can result in reduced secretion efficiencies (Smith *et al*, 1985). This is

not the case with pVT314, as interference in secretion efficiencies alone would presumably be accompanied by accumulation inside the cell. Instead, as with the other constructs, approximately 50-60% of what is produced in pVT314 is secreted, although lower levels are reached overall.

The vector used in constructing pVT314 is similar to pAAH5 and p69D, except for the auxotrophic marker used for selection (uracil in pVT314 and leucine in the pJG series). According to the growth curves shown, higher cell densities are reached in both pJG317 and pJG314 compared to pVT314, and this feature of pVT314 may contribute to the overall lower levels. However, other factors such as glycosylation must also be considered.

Glycosylation appears to interfere with activity of the β-glucanase enzyme and would therefore interfere with the true approximation of β-glucanase production in each strain. This effect would also mask the relative efficiencies of the two signal peptides, since the extent of glycosylation must also be taken into account. If the enzyme is more likely to be glycosylated when attached to the α-factor signal, then the lower level observed in these constructs would be expected. This is also the case if the α-factor signal is more efficient than the bacterial signal at translocating the enzyme. These α-factor signal constructs however, also pose additional problems for β-glucanase when one considers that the signal itself is also glycosylated *in vivo* in yeast. Furthermore, in contrast to the bacterial signal which is cleaved as the enzyme is translocated through the ER membrane, the α-factor signal is retained until the it reaches Golgi.

The sharp increase in extracellular activity seen in the growth curves of pJG314 and pVT314 at approximately 36 hours interesting feature which may reflect processing characteristics of the α-factor signal peptide. This increase is accompanied by a corresponding decrease in activity levels intracellularly and is not apparent in the bacterial signal constructs. α-factor is not processed until the Golgi stage of the pathway and ongoing processing of the intracellular pool of β-glucanase may manifest in this slight delay in secretion of

the enzyme.

(4.5) The effect of glycosylation on the *Bacillus* β-glucanase enzyme.

It is not entirely unexpected that glycosylation could interfere with the activity of a bacterial protein when produced in yeast. Glycosylation of secretory proteins is not a feature of the bacterial secretory process and modifications to areas of a protein that may constitute active sites, can easily be envisaged as interfering with enzyme activty. The evidence from the *sec* mutants, combined with the tunicamycin effect, shows that this is the case for the *B.subtilis* β-glucanase enzyme. The enzyme appears to be less active when modified by glycosylation than in its natural state.

Tunicamycin has a more profound effect on recovery of enzyme activity than production of the enzyme at the restrictive temperature in the glycosylation mutant, *sec*53. As described earlier, tunicamycin inhibits all glycosylation by interfering at the earliest stage in core assembly. The *sec*53 phenotype reduces glycosylation by limiting the number of mannose residues available for incorporation into glycoproteins. This difference could explain the larger increase seen with the inhibition of all sugar additions in the presence of tunicamycin.

Glycosylation has also been shown to interfere with the activity of β-glucanase produced in another *Bacillus* species (*B.macerans*). A reduced specific activity was observed for the glycosylated form when compared to the native form (Oleson and Thomsen, 1991).The glycosylated form of the *B.macerans* enzyme was shown to be more stable than the unglycosylated form. Preliminary analysis of the thermostability of the *B.subtilis* enzyme (not shown) however, suggests the opposite. In this case the unglycosylated form (prepared by treatment with tunicamycin) exhibits a greater tolerance for incubation at high temperatures.

Glycosylation of the *E.coli* β-glucuronidase enzyme in the ER of the tobacco plant has also been shown to interfere with the

activity of this bacterial enzyme (Ittaguria *et al* 1989). Tunicamycin treatment of tobacco cell suspension cultures, carrying the β-GUS gene fused to various lengths of the plant patatin signal sequence, resulted in up to a 100 fold increase in β-glucuronidase activity. Western analysis showed that the enzyme was present at a higher molecular weight than expected in the plant cultures and treatment with tunicamycin or EndoH, reduced it to its normal mature size. In this case, the glycosylation sites occur at amino acid positions 358 and 423. The disruption of activity by glycosylation at these sites, suggests that the active site of the enzyme is toward the carboxy terminus. This is futher substantiated by the *Bal*31 deletion analysis carried out in this study.

Fusion of the β-GUS gene to the α-factor signal peptide in pGUSα6, did not allow secretion of the enzyme out into the yeast culture medium. Also, tunicamycin had no effect on the activity of the enzyme in this strain. Taken in conjunction with Ittaguria's observations, this suggests that the enzyme is not translocated in pGUSα6. Why translocation is prevented may be associated with the large size of the enzyme, in combination with the relatively large signal peptide, presenting steric problems for translocation.

The potential glycosylation sites in the β-glucanase enzyme are more widely spread, occurring at positions 31,38,64 and 184. Activity gel analysis shows that in both pJG317 and pJG314, all of these sites can become modified with core oligosaccharides. However, complete modification does not always occur and a proportion of the enzyme in the secretory pathway is only partly modified at two sites, while a further fraction contains no glycosylation additions. Although most of the secreted fraction in both pJG317 and pJG314 is partially glycosylated to some extent, unglycosylated enzyme is also secreted. This shows that glycosylation is not a prerequisite for secretion of this enzyme in yeast. The extent of the modifications to the higher molecular weight forms of the enzyme, present as a smear in the extracellular samples in pJG317 and pJG314, cannot be examined in this type of zymogram analysis.

Different levels of glycosylation appears to have different effects on the activity of the enzyme. It is obvious that core glycosylation does not completely inhibit β-glucanase activity and it is impossible to tell if the bands visible on activity gels, have reduced enzymatic activity compared to the unmodified bands. It is likely that the core additions do affect activity of the enzyme, but that the inhibition observed is also contributed to by extensive chain additions which have a more severe effect.

Examination of tunicamycin treated β-glucanase in pJG317 and pJG314 confirms that these higher molecular weight bands represent glycosylated forms of the enzyme. In the gel shown in figure (3.27), the higher molecular weight forms are reduced to the size of the unglycosylated band, which can be clearly seen to have increased in intensity. In these supernatant samples, the loadings represent equal levels of β-glucanase activity as determined by DNS assay (ie. a 1/10 dilution of the +tunicamycin sample in pJG317 and a 1/3 dilution of the +tunicamycin sample in pJG314). This dilution was necessary as the large increases in activity caused by tunicamycin treatment resulted in indistinguishable smears of activity running the length of the +tunicamycin lane when equal volumes were added. This underlines the extent of the increase seen and suggests that it is contributed to by previously unseen material, possibly representing highly modified β-glucanase. When cyclohexamide is added to RSY12 cells at the same time as the mutant phenotype is induced, the increases in β-glucanase activity seen at intracellular and extracellular levels are no longer observed (data not shown). Extracellular levels of β-glucanase activity are equal at both 25°C and 37°C. Although cyclohexamide will have halted protein synthesis, the enzyme activity detected extracellularly is presumably derived from the intracellular levels of β-glucanase that was synthesized before induction of the mutant phenotype. This further substantiates the idea that the increases seen at the non-permissive temperature, in the absence of cyclohexamide, is due to the inhibition of oligosaccharide additions to newly

formed β-glucanase.

The smallest glycosylated band representing core oligosaccharide additions at 2 sites, is still visible in the the supernatants after tunicamycin treatment. Because of the nature of this analysis a certain amount of glycosylated enzyme, modified before the addition of tunicamycin and subsequently secreted, is likely to be present and this band may reflect this fraction. In retrospect, this could be eliminated by a short preincubation period in the presence of the drug. A similar band appears in the supernatant of pJG205 cells after tunicamycin treatment. Its slightly faster mobility suggests that it could represent β-glucanase with one core oligosaccharide addition or possibly the enzyme with its signal peptide still intact. Whatever its identity, the band is only evident after glycosylation is inhibited and therefore is derived from β-glucanase material not seen in pJG205 under normal growth conditions.

Why the β-glucanase enzyme produced in yeast is present in a range of different glycosylated forms may be due to the folding properties of the protein as it passes through the ER. Rapid folding may preclude glycosylation, while modification at one site may promote further additions. In pJG317 and pJG205 the proportion of glycosylated material seems to be affected by the amount that is translocated and subsequently secreted. How the majority of the enzyme in pJG205 avoids glycosylation is not clear. Also, the proportion that appears to be glycosylated in this strain seems to have undergone extensive modifications and is only assayable after glycosylation is disrupted. These differences may be associated with the nature of the translocation event and the orientation of the protein passing through the ER membrane when fused to the truncated signal. This limited amount of glycosylation observed in pJG205 can explain the lower increases in activity observed in tunicamycin treated pJG205 compared to tunicamycin treated pJG317 (section 3.11). Where less glycosylation has taken place the inhibitory effect on β-glucanase activity would be expected to be less pronounced.

When compared to pJG317, the effect of tunicamycin in pJG314 is also less dramatic. Inhibition of glycosylation of the α-factor pro-region results in a reduction of α-factor secretion. This is thought to be a result of interference with the folding patterns of the region causing a certain amount of accumulation in the ER (Julius *et al*, 1984b). Reduced secretion of β-glucanase, when fused to the α-factor signal peptide in pJG314, would dilute the extent of the increase expected after tunicamycin treatment in the supernatant of this strain. The resultant accumulation of unprocessed and malfolded enzyme in the lumen of the ER, may also account for the lack of an increase seen at intracellular levels.

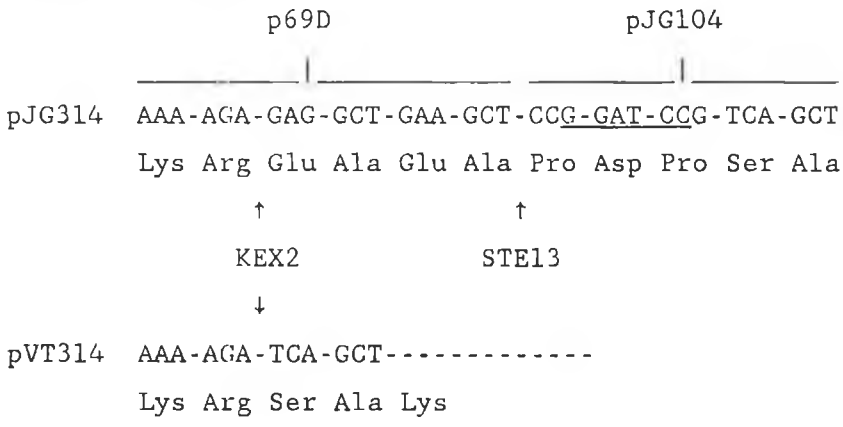
(4.6) **Processing of the α-factor signal peptide in pJG314 and pJG317.**

As outlined in section (1.6.4), processing of the α-factor pheromone protein involves a series of enzymatic cleavage events. The main section of the pro-region is removed after the Lys Arg dibasic cleavage site by the action of the KEX2 protease. The mature portion of the α-factor protein is further processed by the action of STE13 which removes the remaining N-terminal Glu-Ala sequences (Bussey, 1988).

In pJG314, the sequence around the cleavage site of the α-factor pro-region includes these Glu-Ala repeats. A short segment of pUC8 DNA is also included, derived from the *Bam*H1 site used to isolate the signal-less β-glucanase gene from pJG104 (Fig. 4.3). Cleavage at Lys-Arg results in the β-glucanase enzyme retaining 10 extra amino acids at its N-terminus. This is reflected in the slower mobility of the unglycosylated, processed band in the extract of pJG314, seen on the Western blots. The action of the STE13 gene product in α-factor maturation is thought to be the rate limiting factor in secretion of the pheromone (Bussey, 1988). In conjunction with heterologous sequences, cleavage at this site is often inefficient (section 1.7.0) and this seems to be the case in pJG314. The activity gel in Figure (3.25), shows that a small amount of processing by STE13 has occurred with the appearance

of a smaller molecular weight band. In the higher % gel showing the supernatants of pJG314 (Figs. 3.26 and 3.27), the slower mobility of the enzyme is more evident. Also, both of the glycosylated bands are also slightly larger than those seen in pJG317, indicating that the majority of secreted β-glucanase in pJG314 has retained these extra sequences.

Figure (4.3). Sequence around the signal cleavage site in pJG314 and pVT314.



In pVT314, the fusion between the α-factor signal and the β-glucanase gene eliminated these extra sequences, including the STE13 cleavage site (Fig.4.3). By virtue of the *Stu*I site, the Lys Arg cleavage site was fused directly to the the β-glucanase signal-less gene after filling in the *Dde*I overhang. Activity gels suggest that cleavage does occur, producing an enzyme of similar size to pJG317 (Fig.3.28). Heterologous sequences have been known to interfere with cleavage of the α-factor pro-region (section 1.6.4), and inefficient cleavage may contribute to the lower than expected levels of activity seen in this strain. This proposal, however, assumes that retention of the α-factor signal interferes with β-glucanase activity and that inefficient cleavage is a feature of this construct.

The fact that these problems are not apparent in pJG317, may be due to the signal in question being the natural β-glucanase signal sequence. Sequences around the cleavage site are likely to be ideal for processing in *B.subtilis*. That they present no

obvious problem for the yeast signal peptidase, combined with the secretion levels observed, suggests that this signal may be useful for the production of other foreign proteins in yeast. How the signal fares in conjunction with other sequences, however, remains to be seen.

(4.7) **Conclusion.**

The *B.subtilis* β-glucanase enzyme is secreted in yeast using its own bacterial signal peptide. Western analysis showed that the bacterial signal is recognized and cleaved by the yeast signal peptidase. Comparisons to the yeast α-factor signal sequence suggests that the bacterial signal is at least as efficient as the yeast signal peptide. These studies show that the *B.subtilis* signal has definite potential for use as a general secretion signal in yeast. Removal of the bacterial signal peptide resulted in a loss of β-glucanase activity. This was shown to be associated with stability of the enzyme in the yeast cytoplasm and demonstrated the importance of optimizing sequences around the translational initiation site in the generation of fusion constructs. Analysis of a mutant form of the signal peptide showed that a truncated version of the bacterial signal sequence can still translocate β-glucanase, although with reduced efficiency.

Analysis of β-glucanase secretion was obstructed somewhat by the inhibitory effects of glycosylation on activity of the enzyme. On one level this caused problems in assessing the relative efficiencies of the signal peptides used. Glycosylation also interfered with Western analysis in that the antibody raised against the *B.subtilis* β-glucanase enzyme did not recognize glycosylated forms of the enzyme produced in yeast. This is likely to be a result of the widely spread glycosylation sites preventing recognition of the antigenic sites in this relatively small (22KDa) protein. Activity gel analysis circumvented this problem to some extent, but examination of extensively glycosylated forms of the enzyme was still difficult.

Glycosylation also affects the activity of β-glucuronidase, the

other bacterial enzyme examined in this work. This enzyme was chosen for investigation due to its attractive assaying procedures. Although translocation of β-glucuronidase was not achieved in this study, it may be possible to secrete the protein, or a truncated version of the protein, using another signal sequence. The antibody raised against *E.coli* β-glucuronidase is less likely to have problems in recognizing modified forms of the enzyme in yeast than the β-glucanase antibody . The larger size of the protein (72KDa), and the presence of only two glycosylation sites in close proximity to each other, should not prevent recognition of the antibody at other antigenic sites in the enzyme. These glycosylation sites could possibly be removed by the introduction of conservative amino acid substitutions. If this did not interfere with activity of the enzyme, the problem of reduced activity due to glycosylation would also be removed.

CHAPTER 5
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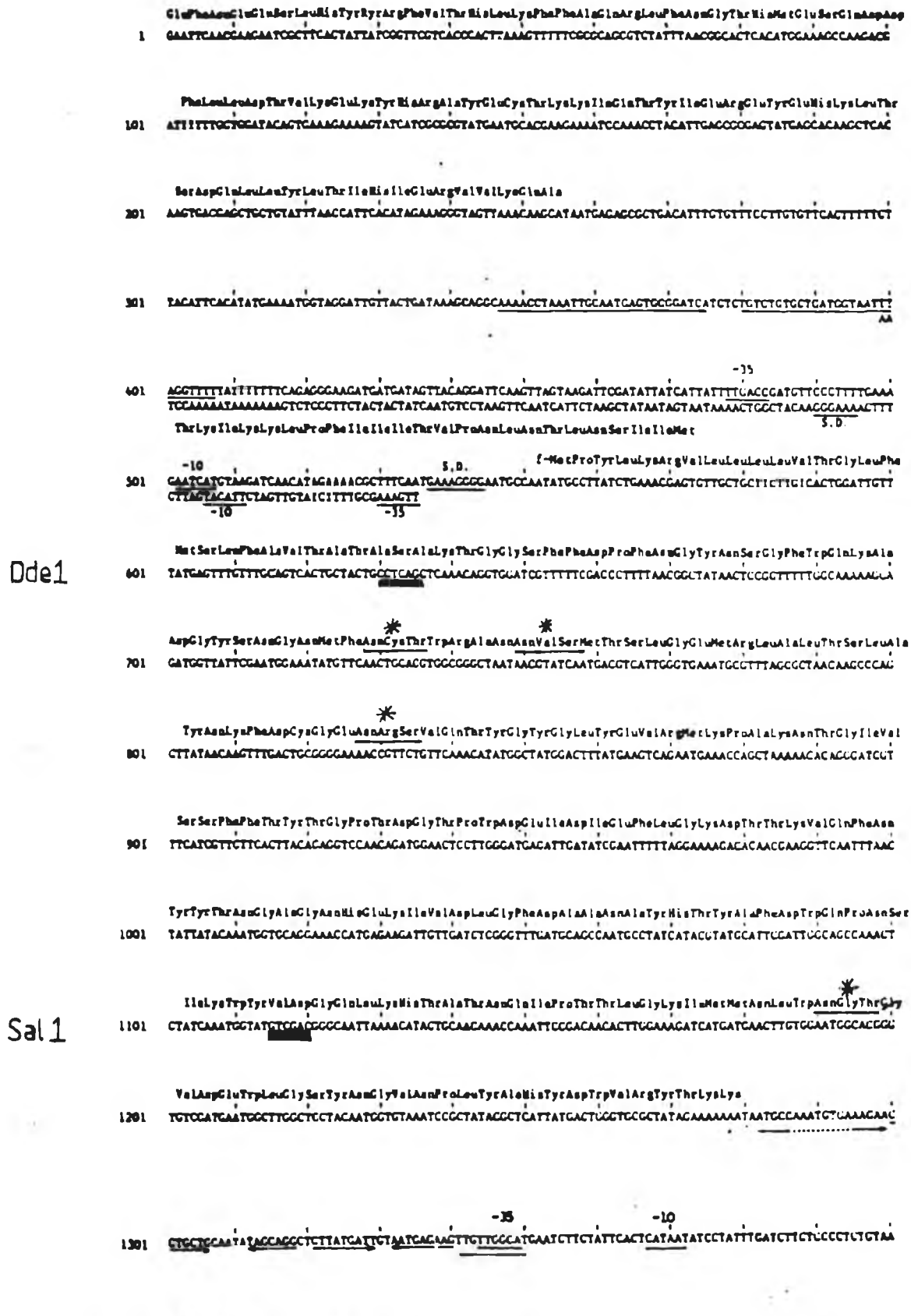
APPENDICES

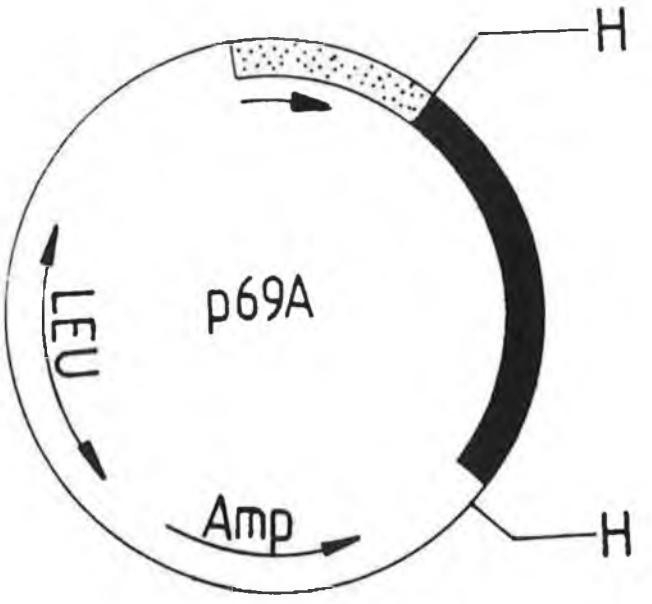
App.1 Sequence of the *Bacillus subtilis* β-glucanase gene (Murphy *et al.* 1984).

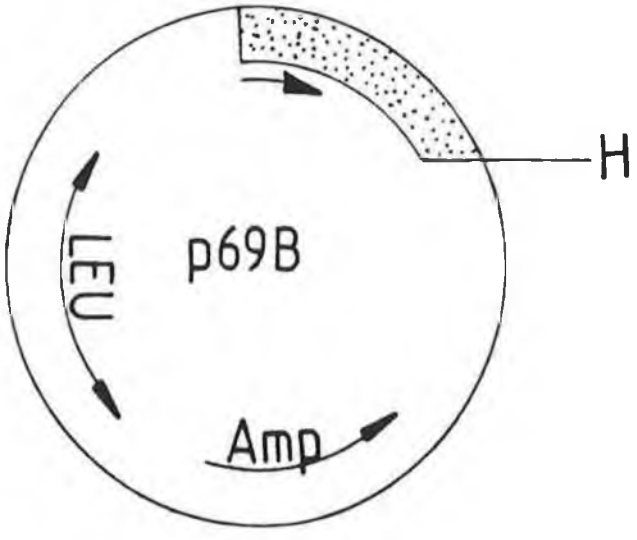
The *Dde*1 site and *Sal*1 site are indicated and glycosylation sites are marked with an asterix.



App.2. Origin of p69A, p69B, p69C, p69D and p69E.

p69A contains the entire α-factor coding sequence, including the signal peptide and structural genes cloned into the *Bam*Hl site of YEp13 (Kurjan and Herskowitz, 1983). The structural genes were removed using the *Hin*dIII restriction site that immediatly follows the signal sequence, to give p69B. p69C, D and E were made by inserting 8', 10', and 12'meric *Bam*HI linkers into the *Hin*dIII site of p69B (T. Rvan D.C.U.).

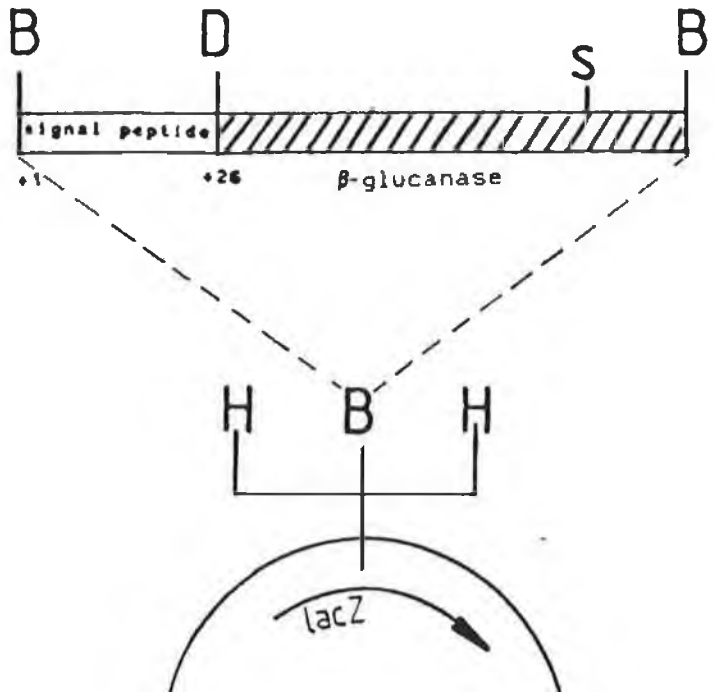


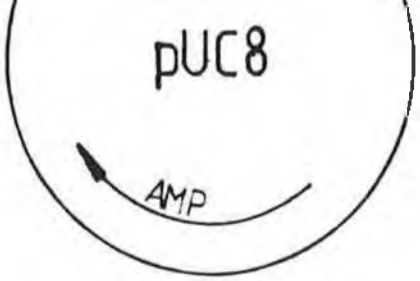


promoter+signal

structural gene

App.3. pJG106.




pUC8
AMP

App.4. pJG104